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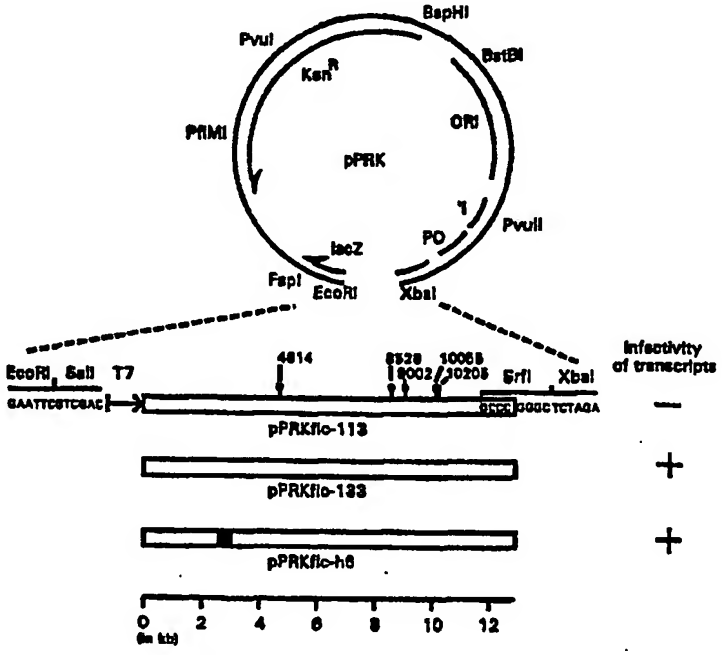
(57) Abstract

The invention provides a nucleotide sequence corresponding to a classical swine fever virus (CSFV) genome or a part or a mutant thereof, which comprises at least a part of the nucleotide sequence of the CSFV C-strain depicted in SEQ ID No. 1, or a complement or RNA equivalent of such nucleotide sequence, or which comprises a nucleotide sequence encoding at least the amino acid sequence 268-494 of the amino acid sequence depicted in SEQ ID No. 1, or a complement or RNA equivalent of such nucleotide sequence. Also provided is a pestivirus polypeptide corresponding to the amino acid sequence 690-1063 of SEQ ID No. 1 or part thereof, which contains a mutation in one of the epitopes within amino acid sequences 691-750 or 785-870, said mutation altering said epitope. Further provided is a method of determining the presence of a test substance capable of specifically binding with a binding site of a binding partner, in a sample, by means of competition of said test substance with a measurable amount of a reference substance capable of specifically binding with the same binding site of said binding partner, comprising: (1) contacting said sample with (a) said reference substance bound to a solid carrier, (b) the binding partner of said reference substance, said binding partner molecule containing at least two identical binding sites for said reference substance, and (c) said reference substance provided with a label; (2) measuring the degree of binding of said label to said carrier.



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Nucleotide sequences of pestivirus strains, polypeptides encoded by these sequences and use thereof for diagnosis and prevention of pestivirus infections.

Field of the invention

The invention discloses a method for the construction of a full-length DNA copy of the genome of the C-strain, a classical swine fever vaccine strain, and transcription of RNA thereof which after transfection in cells gives rise to synthesis of infectious C-strain virus. The invention also comprises C-strain derived (pestivirus) vaccines, as well as subunit vaccines against pestivirus and diagnostic means and methods in relation to pestivirus infections. The invention furthermore provides a method of detecting an immunoactive substance in a sample by means of a competitive assay.

Background of the invention

Classical swine fever (CSF) or hog cholera is a highly contagious and often fatal disease of pigs which is characterised by fever and haemorrhages and can run an acute or chronic course (Van Oirschot. 1986. Hog cholera, p. 289-300. *In* Diseases of Swine. Iowa State University Press, Ames). Outbreaks of the disease occur intermittently in several European and other countries and can cause large economic losses.

Vaccination of pigs with a live attenuated Classical swine fever virus (CSFV) vaccine strain, the "Chinese" strain (C-strain), protects pigs against CSF (Terpstra and Wensvoort. 1988. Vet. Microbiol. 16: 123-128). A major drawback of vaccinating pigs with the conventional vaccines, of which the C-strain is one, is that these vaccinated pigs cannot be distinguished serologically from pigs infected with a CSFV field strain. The C-strain, however, is considered one of the most effective and safe live vaccines. Addition of a (serological) marker to the C-strain would be highly advantageous and would improve the vaccine.

CSFV is a member of the *Pestivirus* genus of the *Flaviviridae* (Francki, R.I.B. et al. 1991. Flaviviridae, p. 223-233. *In* Fifth report of the International Committee on Taxonomy of Viruses. Archiv. Virol. Suppl. 2, Springer Verlag, Vienna). The other two members of the *Pestivirus* genus, which are structurally, antigenically and genetically closely related to CSFV, are Bovine viral diarrhoea virus (BVDV) mainly affecting cattle, and Border disease virus (BDV) mainly affecting sheep (Moennig and Plagemann, 1992. Adv. Virus Res. 41: 53-98; Moormann et al., 1990. Virology 177: 184-198; Becher et al. 1994. Virology 198: 542-551).

The genomes of pestiviruses consist of a positive strand RNA molecule of about 12.5 kb (Renard et al. 1985. DNA 4: 429–438; Moormann and Hulst 1988. Virus Res. 11: 281–291; Becher et al. 1994. Virology 198: 542–551). The positive strand RNA genomes of several non-cytopathogenic BVDV strains, however, may be considerably larger (Meyers et al. 1991. Virology 180: 602–616; Meyers et al. 1992. Virology 191: 368–386; Qi et al. 1992. Virology 189: 285–292).

An inherent property of viruses with a positive strand RNA genome is that their genomic RNA is infectious, i.e. after transfection of this RNA in cells that support viral replication infectious virus is produced. As expected, the genomic (viral) RNA of pestiviruses is also infectious (Moennig and Plagemann, 1992. Adv. Virus Res. 41: 53–98).

For several years recombinant DNA technology has allowed *in vitro* transcription of cloned DNA. This possibility has opened the way to synthesize infectious RNA *in vitro* from a DNA copy of the genome of a positive strand RNA virus. It is well known in the field of molecular engineering that DNA, in contrast to RNA, is easily manipulated by site directed mutagenesis. Hence, the availability of the technique to produce synthetic infectious RNA has greatly enhanced the study of e.g. replication, virulence, pathogenesis, RNA recombination, vector development, and antiviral strategies of the positive strand RNA viruses. However, application of the technology may cause severe problems. The nature of these problems has been described in a recent review by Boyer and Haenni. 1994. (Virology 198: 415–426). In fact, the success or failure to construct a full-length DNA copy of the genome of a positive strand RNA virus and to produce synthetic infectious RNA from such a full-length DNA copy cannot be reliably predicted.

Summary of the invention

The invention provides nucleotide sequences corresponding to a CSFV genome which comprise at least a part of the nucleotide sequence of the CSFV C-strain depicted in SEQ ID No. 1, or a complement or RNA equivalent of such nucleotide sequence, or mutants thereof. Also provided are degenerate nucleotide sequences having different nucleotides but encoding the same amino acids. The invention also covers polypeptides encoded by these nucleotide sequences, and vaccine strains, the genome of which contains such a nucleotide sequence, in particular a recombinant virus strain based on transcripts of a full-length DNA copy of the genome of the CSFV C-strain.

Partial nucleotide sequences as indicated above are also useful, in particular those which contain a mutation in the structural region of the virus genome, i.e. in the nucleotide sequence encoding amino acids 1–1063 of the sequence depicted in SEQ ID

No. 1. The mutation may be a substitution by a corresponding part f the g nome of another pestivirus strain, a substitution of one or amino acids, or a deletion. The mutation may also be an inserted or substituted heterologous nucleotide sequence altering the translation strategy of the CSFV nucleotide sequence or altering the processing of a polypeptide encoded by the CSFV nucleotide sequence. Furthermore, the mutation may be an inserted or substituted heterologous nucleotide sequence encoding a polypeptide inducing immunity against another pathogen; in this case the CSFV sequence is used as a vector for heterologous immunogens.

The invention is also concerned with nucleotide sequences of a pestivirus genome in general or a part or a mutant thereof, which sequences contain a mutation in a subregion of the E1 protein, i.e. in the nucleotide sequence encoding amino acids corresponding to amino acids 691-750 or 785-870 of the sequence depicted in SEQ ID No. 1, as well as the polypeptides encoded by these nucleotide sequences. These polypeptides are particularly useful for protecting animals against a pestivirus infection in such a way so as to allow a diagnosis which distinguishes between animals infected with field strains of the pestivirus and vaccinated animals.

The invention is furthermore concerned with vaccines containing a nucleotide sequence, a polypeptide, or a vaccine strain as indicated above, as well as to diagnostic compositions containing a nucleotide sequence or a polypeptide as mentioned above, or an antibody raised against such polypeptide.

The invention also relates to methods and means for diagnosis of pestivirus infections, especially with such means and methods which distinguish between infected animals infected and vaccinated animals.

The invention also provides a method for determining test substances, such as an antibody or an antigen in an immunoassay, by means of a specific binding test, wherein a specifically binding reference substance in immobilised form and the same specifically binding reference substance in labeled form are used.

Detailed description of the invention

The invention provides the complete cDNA sequence of the RNA genome of the "Chinese" strain (C-strain; EP-A-351901) of CSFV. This allows the construction of a full-length DNA copy of this sequence, of which synthetic RNA can be transcribed that after transfection in suitable cells, such as SK6-M cells (Kasza, L. et al. 1972. Res. Vet. Sci., 13: 46-51; EP-A-351901) gives rise to synthesis of infectious C-strain virus. The use of this finding for the development of modified C-strain vaccines, e.g. vaccines

which contain a (serological) marker, is described. Although the invention is illustrated for one CSFV strain, it is also applicable and useful for other pestivirus strains by exchanging specific genomic segments, described below, between the other pestivirus and the CSFV C-strain, or by constructing an "infectious" DNA copy of the other pestivirus.

The nucleotide sequence of a DNA copy of the genomic RNA of the C-strain is depicted in SEQ ID No. 1. The numerals mentioned in the text are all related to this sequence and may differ slightly in the sequences of other pestiviruses. The nucleotide sequence is 12,311 nucleotides in length and contains one large open reading frame (ORF) of 11,694 nucleotides encoding a polyprotein of 3,898 amino acids. The size of this ORF is the same as that of the genomes of CSFV strains Brescia (Moormann et al. 1990. Virology 177: 184-198) and Alfort (Meyers et al. 1989. Virology 171: 555-567).

The ORF starts with the ATG at nucleotide positions 374 to 376 and stops at the TGA codon at nucleotide positions 12,068 to 12,070. The 5' non-coding region which precedes the ORF is 373 nucleotides in length. Its sequence is highly conserved between strains Brescia, Alfort and C (Fig. 2), and the predicted secondary structure of this region resembles that of the 5' non-coding region of hepatitis C virus (Brown et al. 1992. Nucleic Acids Res. 20: 5041-5045), another member of the *Flaviviridae*. The 5' non-coding region of hepatitis C virus has been shown to contain an internal ribosome entry site (Tsukiyama-Kohara et al. 1992. J. Virol. 66: 1476-1483). Such sites have important regulatory functions (Agol. 1991. Adv. Virus. Res. 40: 103-180). The analogy with hepatitis C virus indicates that the 5' non-coding region of CSFV also contains an internal ribosome entry site, which is located approximately between nucleotides 124 and 374 of the sequence of SEQ ID No. 1, as important regulatory element. The internal ribosome entry site may be used as a site for mutation in order to attenuate the virus, as well as for altering the translation strategy of the ORF.

A second important region regulating replication of pestiviruses is the 3' non-coding region. Upon alignment of the C-strain sequence with the sequences of strains Brescia and Alfort, a sequence of 13 nucleotides unique to the C-strain was observed in this region (Figure 2B). This unique sequence TTTTCTTTTTTTT is located from nucleotide positions 12,128 to 12,140 in the sequence of SEQ ID No. 1. It is the only insertion of more than two nucleotides in a row observed in the sequence of the C strain compared to the sequences of strains Brescia and Alfort. For the rest, the sequences in the 3' non-coding regions of three CSFV strains are highly homologous. The overall homology between sequences in this region is lower when CSFV strains and BVDV

strains are compared. Nevertheless, it is clear that the TTTTCTTTTTTT sequence of the C-strain is also absent in the sequences of the 3' non-coding regions of the BVDV strains. The TTTTCTTTTTTT sequence therefore appears to be unique to the genome of the C-strain, and will provide an excellent marker for a C-strain specific sequence. This sequence can be used as a basis for nucleotide probes, and for sequence determination, to identify C-strain specific pestiviruses. Therefore, all pestivirus strains having this sequence in their 3' non-coding region (not necessarily at an identical position as in the C-strain) are considered related to the C-strain, and are also part of the invention.

A crucial parameter for infectivity of transcripts of a DNA copy of the genome of a pestivirus is the amino acid sequence. In this respect, two aspects regarding the cloning and sequencing of RNA viruses in general, and pestiviruses in particular, had to be considered. First, the mutation frequency of the genome of positive strand RNA viruses is high (about $1/10^4$ nucleotides during replication), and therefore no stock of virus or viral RNA preparation is ever clonal with regard to the viral RNA it contains. Among these RNA molecules there may also be molecules which are noninfectious. If this were caused by premature stop codons in the large open reading frame, this would be easily recognised. Also mutations affecting active sites of viral enzymes, or known structures of proteins would be recognizable. However, where the relation between the amino acid sequence and the function and structure of a protein is unknown, which is the case with most of the pestivirus proteins, it is impossible to predict which amino acid is valid and which one is not. Second, mutations may have been introduced during cDNA synthesis. Therefore, the genome of the C-strain was cloned and sequenced independently twice. Regions with discrepancies between the sequences were cloned and sequenced at least thrice (compare Figure 1). The sequence which was encountered twice at a particular position was regarded as the correct one at that position. The necessity of this approach for the generation of infectious transcripts of a DNA copy of the genome of the C-strain is demonstrated by the following finding. Full-length DNA copy pPRKflc-113, composed after the second round of cloning and sequencing (Fig. 3), appeared to be noninfectious. After cloning and sequencing of regions with discrepancies between the sequences of cDNA clones of the first and second round, there appeared to be five amino acids which were different in the full-length copy of the second round cDNA clones and the sequence of the C-strain considered correct. After correction of these five amino acids in pPRKflc-113, clone pPRKflc-133 was obtained which generated infectious transcripts (Figure 4). The 5 differences are located at amino acid positions 1414 (Val→Ala); 2718 (Gly→Asp); 2877 (Val→Met); 3228 (Leu→Met);

3278 (Tyr→Glu). Th amino acids ncoded at these positions by the cDNA sequence which is noninfectious are indicated before the arrow, amino acids at these positions in the copy that is infectious are indicated after the arrow (SEQ ID No. 1). Whether each of the amino acid changes individually will abolish infectivity of the C-strain DNA copy will have to be determined by analysing infectivity of transcripts with individual mutations of each of the five amino acids. However, this finding shows that small differences in the amino acid sequence may be crucial for infectivity of transcripts of a DNA copy of the genome of the C-strain. It also indicates that preparing infectious transcripts of a copy of the sequence of a pestivirus may in practice appear to be impossible because of small differences in sequences (even at the one amino acid level) which may go unnoticed.

C-strain derived mutants that are suitable for (marker) vaccine development are part of the invention. They may contain mutations like deletions, insertions, (multiple) nucleotide mutations, and inserted and/or exchanged genomic fragments originating from other pestivirus strains, in the nucleotide sequence described in SEQ ID No. 1.

The sequence of the C-strain can be divided in four regions suitable for mutation and/or exchange. Region one is the 5' non-coding sequence running from nucleotides 1 to 373. Region two encodes the structural proteins N^{pro}-C-E2-E3-E1 and runs from nucleotides 374 to 3563. Region three encodes the nonstructural proteins and runs from nucleotides 3564 to 12068. Region four is the 3' non-coding sequence which runs from nucleotides 12069 to 12311.

One region that is particularly suitable for making C-strain marker vaccines comprises the genomic region encoding the structural proteins N^{pro}-C-E2-E3-E1. This region is located between amino acids 1 and 1063 in the sequence of SEQ ID No. 1. Preferred subregions of this part of the genome are specified by the following amino acid sequences 1-168 (N^{pro}), 169 to 267 (C), 268 to 494 (E2), 495 to 689 (E3), and 690 to 1063 (E1), or parts thereof. As an example the N-terminal antigenic part of the region encoding E1 of the C-strain, running from amino acid 690 to 877, was exchanged with the corresponding region of E1 of strain Brescia (Figure 4, pPRKflc-h6). The newly generated C-strain derivative is infectious and can be discriminated from the wild-type strain and from strain Brescia through reaction with C-strain and Brescia specific monoclonal antibodies, directed against E1 and E2; as an example, the resulting C-strain reacts with monoclonal antibodies specific for E1 of strain Brescia (Table 1). Thus, the antigenic properties of this new mutant have changed with respect to the parent virus, demonstrating that xchanging the N-terminal half of E1 of the C-strain with that of another CSFV strain is one approach to the development of a C-strain marker vaccine.

However, the invention is not restricted to exchange of N-terminal halves f E1 between the C-strain and other CSFV strains. The N-terminal halves of E1 from any other pestivirus strain may be exchanged with corresponding parts of E1 of the C-strain. In this respect, E1 sequences of pestivirus strains which are isolated from pigs, but belong to an antigenic group other than the C-strain, are particularly suitable. Examples of such strains, which were selected on the basis of cross-neutralisation, include strains "Van EE", "Stam", "SF UK 87", "Wisman", and "5250" (Wensvoort et al. 1989. Vet. Microbiol. 20: 291-306; Wensvoort. 1992. *In*: Report on meeting of national swine laboratories within the European Community. 16-17 june 1992. VI/4059/92-EN(PVET/EN/1479) 1992, p59-62).

The N-terminal half of E1 has been shown to contain three distinct antigenic domains, A, B and C, located on distinct parts of the E1 protein and each reacting with strongly neutralizing monoclonal antibodies (Wensvoort. 1989. J. Gen. Virol. 70: 2865-2876; Van Rijn et al. 1992. Vet. Microbiol. 33: 221-230; Van Rijn et al. 1993. J. Gen. Virol. 74: 2053-2060). Epitopes conserved among 94 CSFV strains tested, map to domain A, whereas the epitopes of domains B and C are non-conserved (Wensvoort. 1989. J. Gen. Virol. 70: 2865-2876). Mapping of epitopes with hybrids of the E1 genes of strains Brescia and C (Van Rijn et al. 1992. Vet. Microbiol. 33: 221-230), and with deletion mutants of E1 of strain Brescia, suggest that domains A and B + C form two distinct antigenic units in the N-terminal half of E1 (Van Rijn et al. 1993. J. Gen. Virol. 74: 2053-2060). This suggestion was further supported by the finding that the six cysteines located at positions 693, 737, 792, 818, 828, and 856, in the N-terminal half of E1 are critical for the correct folding of E1. However, at least Cys 792 is not crucial for infectivity of strain Brescia, because a monoclonal antibody resistant mutant of this virus was isolated with a Cys→Arg mutation at this position (Van Rijn et al. 1993. Presentation and abstract at the 9th International Congress of Virology, 8-13 August, Glasgow, Scotland).

Whereas small changes in the amino acid sequence may abolish infectivity of the RNA of the C-strain (see Example 2), the cysteine change at position 792 shows that an amino acid change at a position which is less predicted to be suitable for modification without loss of function, may still result in a viable virus mutant. Thus, the effects of a particular amino acid change on the properties of the virus will have to be determined empirically for each amino acid in the sequence of strain C. This again shows that no obvious target sequences for modification of the C-strain, e.g. for marker vaccine development, can b identified on the basis of previously published information.

Essential to the development of C-strain marker vaccines is the possibility to differentiate serologically between vaccinated pigs and pigs infected with a CSFV field strain. It was shown previously that a live attenuated pseudorabies virus vector expressing E1, or immunoaffinity purified E1, expressed in insect cells with a baculovirus vector, induces a protective immune response in pigs against hog cholera (WO 91/00352; Van Zijl et al. 1991. J. Virol. 65: 2761-2765; Hulst et al. 1993. J. Virol. 67: 5435-5442). It was surprisingly found that mutants of E1 with a deleted A domain or with deleted B + C domains (Figure 5), also induce a protective immune response in pigs against hog cholera (Table 2). This indicates that protective immunity induced by the vaccine strain does not depend on neutralizing antibodies against both domains A and B + C. Therefore, pestivirus strain mutants having exchanged or mutated only the A domain, or only the B + C domains, or parts thereof, with the corresponding region of another pestivirus, preferably but not exclusively a pestivirus isolated from pigs belonging to a different antigenic group than the C-strain (for examples see above), are also part of the invention. The region of E1 covering domain A and suitable for exchange or mutation, is located between amino acids 785 and 870. Parts of this region may also be suitably exchanged or mutated, e.g. the subregions located between amino acids 785 and 830 and between amino acids 829 and 870. The region of E1 covering domains B + C and suitable for exchange or mutation is located between amino acids 691 and 750. Parts of this region may also be suitably exchanged or mutated, e.g. the subregions located between amino acids 691 and 718 and between amino acids 717 and 750.

Animals infected with pestiviruses develop antibodies against E2 (Kwang et al., 1992. Vet. Microbiol. 32: 281-292; Wensvoort. unpublished observation). Therefore, a second region suitable for (marker) vaccine development via mutation (deletions, insertions, point mutations), or exchange of corresponding genetic material with an antigenically different pestivirus, or with a pestivirus belonging to a different antigenic group, is the region encoding E2.

The C-strain may also be used as a vector for the insertion and expression of heterologous genetic material (sequences). For vector development, heterologous genetic material inserted into the C-strain serves to alter translation strategy of the large ORF and processing of the polyprotein encoded by this ORF. An example of a sequence suitable for altering the translation strategy of the large ORF is a sequence specifying an Internal Ribosome Entry Site (IRES) (Duke et al. 1992. J. Virol. 66: 1602-1609, and references therein). An example of a sequence suitable for altering processing of the polyprotein is a signal sequence responsible for translocation of proteins exported from

the cell or inserted into membranes, across th membrane of the endoplasmatic r_ticulum (Blobel. 1980. Proc. Natl. Acad. Sci. U.S.A. 77: 1496–1500; Kreil. 1981. Annu. Rev. Biochem. 50: 317–348). Signal sequences are cleaved by cellular signal peptidases. However, sequences encoding cleavage sites of viral proteases may as well be used to alter processing of the polyprotein.

Sequences inserted and expressed by a C-strain vector may be used as a marker to identify vaccinated pigs, or may be used to protect pigs against the pathogen from which the heterologous inserted sequence originates. Marker sequences are preferably highly antigenic and belonging to microorganisms not replicating in pigs. They may encode known complete gene products (e.g. capsid or envelope proteins) or antigenic parts of these gene products (e.g. epitopes). Preferably marker sequences originate from viruses belonging to the families: *Adenoviridae, Arenaviridae, Arteriviridae, Bunyaviridae, Caliciviridae, Circoviridae, Coronaviridae, Flaviviridae, Hepadnaviridae, Herpesviridae, Orthomyxoviridae, Paramyxoviridae, Papovaviridae, Rhabdoviridae, Parvoviridae, Poxviridae, Picornaviridae, Reoviridae, Retroviridae, and Togaviridae.* However, marker sequences may also encode artificial antigens not normally encountered in nature, or histochemical markers like *Escherichia coli* β-galactosidase, *Drosophila* alcohol dehydrogenase, human placental alkaline phosphatase, firefly luciferase, and chloramphenicol acetyltransferase.

Heterologous genetic material encoding one or more proteins inducing protective immunity against disease caused by the pathogen corresponding with the heterologous genetic material may be derived from other pestivirus strains, including sequences of strains specified above, porcine parvovirus, porcine respiratory coronavirus, transmissible gastro–enteritis virus, porcine reproductive and respiratory syndrome virus (Lelystad virus, EP. 92200781.0), Aujeszky's disease virus (pseudorabies virus), porcine endemic diarrhoea virus, and porcine influenza virus, and bacteria, such as *Pasteurella multocida, Bordetella bronchiseptica, Actinobacillus pleuropneumoniae, Streptococcus suis, Treponema hyodysenteria, Escherichia coli, Leptospira*, and mycoplasmata, such as *M. hyopneumoniae* and *M. hyorhinis.*

Suitable sites for insertion of heterologous sequences in the C-strain, but not the only ones, are located between amino acid residues 170 and 171, between residues 690 and 691, and between residues 691 and 692 and are indicated in SEQ ID No. 1.

The invention also includes diagnostic tests which can be used to discriminate between pigs vaccinated with a marker vaccine, or a subunit vaccine containing (mutated) E1 and/or (mutated) E2, and pigs infected with a pestivirus field strain.

Suitable forms of such differential diagnostic tests ar described in Examples 4 and 5. In the conventional non-discrimiminatory CSFV ELISA test, E1 is used as antigen in the complex trapping blocking (CTB) ELISA assay described by Wensvoort et al., 1988. (Vet. Microbiol. 17: 129-140). This prior art CTB-ELISA, also called Liquid Phase Blocking ELISA, or double antibody sandwich ELISA, uses two monoclonal antibodies (Mabs) which were raised against E1 of CSFV strain Brescia. The epitope for Mab b3, which is located within domain A, is conserved among CSFV strains, whereas the epitope of Mab b8, which is located within domain C, is nonconserved (Wensvoort. 1989. J. Gen. Virol. 70: 2865-2876). The above CTB-ELISA is sensitive, reliable and specifically detects CSFV specific antibodies in pigs infected with a pestivirus. Thus, the test differentiates between pigs infected with a CSFV strain and pigs infected with e.g. a BVDV strain. However, the test does not differentiate between pigs infected with a CSFV field strain and pigs vaccinated with the C-strain vaccine. Also this test is not suitable in conjunction with an E1 subunit vaccine whether live or dead.

One test according to the invention is a modified CTB-ELISA, based on only one MAb, e.g. MAb b3. Such a CTB-ELISA, based on only one Mab which Mab is used for binding of the antigen to the surface of an ELISA plate as well as competition with a field serum has not yet been described and is an essential part of this invention. Now that the principle of this test has been described, it can be usefully applied to the development of diagnostic kits for the detection of other antibodies including antibodies against other viruses or other diseases, or antibodies which are indicative for other conditions of the human or animal body. The finding is therefore useful for all CTB-ELISA's, or ELISA's based on the same principle as a CTB-ELISA, which are developed on the basis of a single Mab and a dimerised or multimerised antigen. The claimed test method is also applicable to the determination of other members of pairs of specifically binding partner molecules, such as activators/receptors, enzymes/inhibitors and the like, wherein one of the partners has at least two identical binding sites.

Thus the invention also comprises a method of determining the presence of a test substance (e.g. antibody) capable of specifically binding with a binding site of a binding partner (e.g. antigen), in a sample, by means of competition of said test substance with a measurable amount of a reference substance (antibody) capable of specifically binding with the same binding site of said binding partner, comprising

(1) contacting said sample with (a) said reference substance (antibody) bound to a solid carrier, (b) the binding partner (antigen) of said reference substance, said binding partner molecule containing at least two identical binding sites for said reference substance, and

(c) said reference substance (antibody) provided with a label;

(2) measuring the degree of separation of said label from said carrier.

As an example, said binding partner (antigen) to said reference substance (antibody), containing at least two identical binding sites is a dimer of a binding partner (antigen) to said reference substance.

Using the same principle, the invention also comprises a method of determining the presence of a test substance (antigen) having at least two identical binding sites per molecule for specifically binding with a binding partner (antibody), in a sample, comprising

(1) contacting said sample with (a) said binding partner (antibody) bound to a solid carrier, and (b) said binding partner (antibody) provided with a label;

(2) measuring the degree of binding of said label to said carrier.

In these methods, the antibodies and antigens are only referred to by way of example; they may be substituted by other specifically binding partner molecules.

Further provided is a diagnostic kit containing: (a) a reference monoclonal antibody bound to a solid carrier, (b) said reference monoclonal antibody provided with a label; and optionally (c) an antigen to said reference antibody containing at least two identical binding sites for said reference antibody; or a complex between said components (a) and/or (b) and (c); as well as further components for carrying out a competitive immunological assay.

The method is suitable as a differential diagnostic test in conjunction with an E1 subunit vaccine, which has a deletion in one or more epitopes of E1, e.g. domain A. The test is also suitable in conjunction with subunit E1 of which the A domain has been mutated such that antibodies induced against such mutated A domain do not compete with Mab b3 for the epitope of Mab b3. Furthermore, the test is suitable in conjunction with a modified C-strain or other CSFV strain vaccines with a deletion in domain A, with a domain A which has been exchanged with that of a pestivirus belonging to a different antigenic group as CSFV (see above), or with a domain A which has been mutated such that antibodies directed against that domain do not compete with Mab b3 for the epitope of Mab b3. Although the test is described and exemplified for domain A of E1, a similar test based on only Mab b8 can be used in conjunction with a vaccine with a deletion in domains B + C or domain C, with domain B + C or domain C which has been exchanged with that of a pestivirus belonging to a different antigenic group as CSFV (see above), or with domain B + C or domain C which has been mutated such that antibodies directed against those domains do not compete with Mab b8 for the epitope of Mab b8. The test

is illustrated in conjunction with Mab b3 or Mab b8 of strain Brescia. However, the test may be usefully set up with other Mabs direct d against domain A or domains B + C of E1 of strain Brescia or against domain A or domains B + C of any other CSFV strain, but also with Mabs against analogous domains in E1 of any other pestivirus. The test can also be based on epitopes of E2 (see Example 5). Antigens suitable in the (modified) CTB-ELISAs according to the invention are preferably dimers or multimers of E1 (plus or minus a 3'-TMR) or E2 (see Example 5) of CSFV strains reacting with Mab b3 or Mab b8 or similar MAbs directed against E2 epitopes. In the case of a vaccine with a mutated A domain, dimers or multimers of the antigen used for the diagnostic test may be synthesised by the deletion B + C construct (see Example 5), or in the case of a vaccine with mutated B + C domains, dimers or multimers of the antigen used for the diagnostic test may be synthesised by the deletion A construct (compare Figure 5 for constructs; compare Examples 4 and 5). The dimerised (or multimerised) form of the E1 antigen is believed to be based on disulphide bridges formed by cysteine residues in the C-terminal part of E1. It allows a very sensitive immunoassay, as the dimerised antigen molecule contains two copies of the epitope of one Mab. Thus, this one Mab can be used for immobilising the dimerised antigen via one epitope, and for labeling the dimerised antigen via the other epitope. Competition by sample serum antibodies raised as a result of field strain infection inhibits binding of the labeled antibody to the antigen, and thus results in a sensitive test for the presence of such antibodies. The invention also relates to diagnostic kits based on this method, which kit comprises E1- or E2-based antigens, and (enzyme-) labeled and immobilised monoclonal antibodies of the same type directed at an E1 or E2 epitope, as well as further conventional components (plates, diluents, enzyme substrate, colouring agents, etc.) for carrying out an immunoassay of the competition type.

The vaccine according to the invention contains a nucleotide sequence as described above, either as such or as a vaccine strain or in a vector or host organism, or a polypeptide as described above, in an amount effective for producing protection against a pestivirus infection. The vaccine can also be a multipurpose vaccine comprising other immunogens or nucleotides encoding these. The vaccines can furthermore contain conventional carriers, adjuvants, solubilizers, emulsifiers, preservatives etc. The vaccines according to the invention can be prepared by conventional methods.

The method of the invention for the production of infectious transcripts of a full-length DNA copy of the genome of a CSFV strain, the C-strain, is useful for any other C-strain derived, or pestivirus strain. The method, described here for a live attenuated

CSFV vaccine strain, may also be very usefully applied to *in vitro* attenuate (modify) the C-strain or any other CSFV or pestivirus strain, for vaccine purposes.

The C-strain vaccine according to the invention allows serological discrimination between vaccinated pigs and pigs infected with a CSFV field strain. Marker vaccines of any other CSFV-strain or pestivirus strain may equally well be obtained using the methods of the invention. Such marker vaccines may be developed for instance by mutating (deletions, point mutations, insertions) the region encoding E1, or the N-terminal half of E1, or domains A or B + C of E1, or the region encoding E2 of the C-strain, or analogous regions in the genomes of C-strain derived, or other pestivirus strains, or by exchanging these regions with the corresponding regions of antigenically different pestiviruses or of pestiviruses belonging to a different antigenic group.

An alternative approach to the development of a C-strain marker vaccine is to add to its genome heterologous genetic material expressing a highly antigenic protein or epitope(s) of a microorganism not replicating in pigs, or of artificial nature and not normally occurring in pigs.

Furthermore such heterologous genetic material may encode antigens inducing protective immunity against a disease caused by a microorganism pathogenic for pigs. Therefore, application of the C-strain, or strains derived from the C-strain, or whatever other pestivirus strain, as a vector for the expression of heterologous antigens inducing protection against a particular disease in a host organism, the host organism being a mammal, is also part of the invention. The construction of recombinant C-strain viruses expressing heterologous sequences and suitable sites for insertion of these heterologous sequences are described above. Analogous recombinant viruses can be made for C-strain derived viruses, or for any other pestivirus. These viruses are therefore also part of the invention.

An essential part of the invention relates to the immunogenic potential of subunit E1 with deletions in domain A, or domains B + C. As summarised in Table 2, both of these mutant E1s are capable of inducing protective immunity in pigs against challenge with a lethal dose of the highly virulent Brescia strain. The use of mutants of E1 containing deletions or other mutations in domains A and B + C as dead subunit vaccine, or as live subunit vaccine expressed by a vector system in the vaccinated animal, against CSF, is also part of the invention. Also mutated E1 together with other antigenic CSFV proteins, e.g. E2 or a mutated form of E2, is suitable as dead or live subunit vaccine (see above).

The invention also includes diagnostic tests which can be used to discriminate between pigs vaccinat d with a CSFV marker vaccine, or a subunit vaccine containing (mutated) E1 and/or (mutated) E2, and pigs infected with a pestivirus field strain. Such a diagnostic test may be based on serology, antigen detection, or nucleic acid detection. The choice which test is appropriate in a given case is amongst others dependent on the specificity of the marker used. One suitable form of a serological diagnostic test is the modified CTB-ELISA, described in example 4. According to the invention, this method, based on a CTB-ELISA using a single antibody, is not restricted to the use in the context of CSFV or other pestiviruses, but is also applicable to the determination of other antibodies for other diagnostic purposes in the human or animal field, as well as to the determination of other specifically binding substances.

An example of a suitable antigen detection test in conjunction with a C-strain marker vaccine is a test detecting CSFV field strain E1 and not vaccine strain E1 in the blood of pigs. If the A domain of the C-strain has been modified by e.g. exchange of this domain with that of a pestivirus strain belonging to a different antigenic group than CSFV, such a test may be based on monoclonal antibodies recognizing conserved epitopes of the A domain of CSFV.

However, if the E2 region of the C-strain is modified for marker vaccine development, a serological or antigenic diagnostic test accompanying such a vaccine detects differences between vaccinated and infected animals, in relation to the modified E2 region. Such a diagnostic test thus uses E2 specific sequences as an antigen. These E2 specific sequences may originate from the parent C-strain (see example 5), from CSFV strains which are antigenically different from the C-strain, or from pestiviruses belonging to a different antigenic group than CSFV. However, these E2 specific sequences may also be obtained via mutation (deletion(s), insertion(s), or point mutation(s)) of native E2 of any pestivirus, or may consist of (mutated) parts of E2 of any pestivirus. Dimeric E2 and multimeric E2 may be used as antigen in a diagnostic test (see example 5). Also E2 in conjunction with one monoclonal antibody (compare Examples 4 and 5) may be used in a CTB-ELISA test, the principle of which has been described above. A diagnostic test based on E2 is described in Example 5. Where an antigen detecting kit is to detect pestivirus E2 and is based on one Mab, such test kist preferably contains an antibody recognising a conserved epitope on E2. Such tests are also part of the invention.

Finally, a diagnostic test may be based on the specific detection of a region of CSFV field strains which is modified in the C-strain. Suitabl techniques for this test includ nucleic acid hybridisation, .g. with specific probes, and/or amplification, e.g. with

the polymerase chain reaction. Alternatively, C-strain sequences may be distinguished from CSFV field strain sequences by PCR amplification of (a part of) the 3' non-coding region containing the TTTTCTTTTTTTT sequence unique to the C-strain genome.

If the C-strain is modified by insertion of a heterologous marker sequence, any form of a diagnostic test based on this sequence, e.g. based on the antigen, epitope(s), or histochemical product encoded by this sequence, or based on detection of the heterologous genetic information via nucleic acid hybridisation techniques, e.g. specific probes, and/or amplification techniques, like the polymerase chain reaction, is also part of the invention.

Example 1

Molecular cloning and sequencing of the genome of the C-strain.

Cells and virus Swine kidney cells (SK6-M, EP-A-351901) were grown in Eagle's basal medium containing 5% fetal bovine serum (FBS) and antibiotics. FBS was tested for the presence of BVDV and BVDV antibodies as described (Moormann *et al.* 1990. Virology 177: 184-198). Only sera free from BVDV and BVDV antibodies were used.

The "Chinese" vaccine strain (C-strain) of Classical swine fever virus (CSFV) was adapted to SK6-M cells as described in EP-A-351901. The strain designated "Cedipest" is noncytopathic and was biologically cloned by threefold endpoint dilution. After three amplification steps a cloned virus stock with a titer of $3{,}5.10^6$ $TCID_{50}$/ml was produced.

Isolation of cytoplasmic RNA of SK-6 cells infected with the C-strain.

Intracellular RNA from cells infected with the C-strain was isolated essentially as described (Moormann *et al.* 1990. Virology 177: 184-198).

Briefly, monolayers of SK6-M cells in 162 cm^2 bottles (Costar) were infected with Cedipest at a multiplicity of infection (m.o.i.) of 5 $TCID_{50}$ per cell. Subsequently, cells were incubated for 1.5 hr at 37°C, and fresh medium was added to a final volume of 40 ml. After 7 hrs Actinomycin D was added to a final concentration of 1 µg/ml. After 24 hrs cells were washed twice with ice cold phosphate buffered saline (PBS), and lysed in ice-cold lysisbuffer (50 mM Tris-HCl pH 8.2, 0.14 M NaCl, 2 mM $MgCl_2$, 5 mM DTT, 0.5% [v/v] NP-40, 0.5%[w/v] Na-deoxycholate, and 10 mM vanadyl ribonucleoside complexes (New England Biolabs)). The lysates were centrifuged (4°C, 5 min., 4000 *g*) and the supernatant was treated with proteinase K (250 µg/ml, final concentration) for 30 min. at 37°C, extracted twice with phenol, chloroform, and isoamyl alcohol (49:49:2), and extracted once with chloroform and isoamyl alcohol (24:1). RNA was stored in ethanol.

Synthesis and amplification of cDNA.

One to two μg of cytoplasmic RNA of cells infected with th C-strain, and 20 pmol (-)sense primer were incubated with 1 μl 10 mM methylmercury hydroxide for 10 min. at room temperature. The denaturated RNA was then incubated with 1 μl 286 mM ß-mercaptoethanol for 5 min. at room temperature. The RNA was reverse transcribed with 200-400 units M-MLV reverse transcriptase deficient of RNase H (Promega) for 45 min. at 42 °C in 1x M-MLV reverse transcriptase buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT), 40 U rRNasin (Promega), and 80 μM of dATP, dGTP, dCTP and dTTP. The final reaction volume was 25 μl. The samples were overlaid with 30 μl of mineral oil (Sigma).

After reverse transcription (RT) the samples were denaturated for 10 min. at 94°C. Portions of 2.5 μl of each RT-reaction were amplified in a polymerase chain reaction (PCR) of 39 cycles (cycle: 94°C, 60 sec.; 55°C, 60 sec. and 72°C, 1-2 min.) in 100 μl Taq polymerase buffer (supplied by the manufacturer of Taq polymerase) containing 1 μM of the (+) as well as the (-) sense primer, 200 μM of each of the four dNTPs, and 2.5 U Taq DNA polymerase (Boehringer Mannheim). The samples were overlaid with 75 μl of mineral oil (Sigma).

Cloning of cDNA covering the complete genome of the C-strain.

The genome of the C-strain was cloned indepently twice. During the first round of cloning (Figure 1A), primers for first strand cDNA synthesis and PCR were selected on the basis of homology between the sequences of the CSFV strains Brescia (Moormann *et al.* 1990. Virology **177:** 184-198) and Alfort (Meyers et al. 1989. Virology **171:**555-567), and the BVDV strains Osloss (Renard et al. EP 0208672) and NADL (Collett et al. 1988. Virology. **165:** 191-199). The sizes of the cDNA fragments were chosen between 0.5-2.5 kb in order to obtain optimal amplification. Gel purified amplification products were treated with T4 DNA polymerase and Klenow DNA polymerase I, and phosphorylated with T4 polynucleotide kinase. Thereafter, cDNA fragments were ligated with T4 ligase into the SmaI site of pGEM4z-blue.

In the second round of cloning (Figure 1B), primers were selected from the sequence of the cDNA clones obtained after the first round of cloning. Where possible, primers contained restriction sites suitable for cloning of the amplified cDNA fragments. After RT and PCR amplification (see above), cDNA fragments were either cut with two different restriction enzymes, or blunted and phosphorylated (as described above) at one end, and digested with a suitable restriction enzyme at the other end. If it was not

possible to use PCR introduced restriction sit s located in the primers, a site within the amplified cDNA fragment was chosen for cloning. After gel purification, PCR products were ligated into gel purified pGEM4z-blue (Promega) or pGEM5zf(+) (Promega), digested with restriction enzymes creating ends compatible with those of the PCR products.

To obtain cDNA clones containing the ultimate 5' and 3' ends of the genome of the C-strain, we used the 3'-5' ligation method (Mandl *et al.* 1991. Journal of Virology 65:4070-4077). Cytoplasmic RNA was isolated from cells infected with the C-strain as described above, and was further purified through a 5.7 CsCl cushion (Moormann and Hulst. 1988. Virus Res. 11: 281-291). Based on results suggesting that there is no Cap structure at the 5' end of the BVDV genome (Brock *et al.* 1992. J. Virol. Meth. 38: 39-46), genomic RNA of the C-strain was ligated without previous treatment with pyro-phosphatase. Eight μg of RNA was ligated in a reaction mix of 50 mM Tris-HCl pH 8.0, 10 mM MgCl2, 10 mM DTT, 20 U rRNasin (Promega), 10 μg/ml BSA (RNase free) and 1 mM ATP, using 10 U of T4 RNA ligase (New England Biolabs). The mixture was incubated for 4 hrs at 37°C. RNA was extracted with phenol/chloroform, precipitated with ethanol, pelleted, and resuspended in RNase-free water. Portions of 2 μg RNA were reverse transcribed and amplified as described above. Portions of 2 μl of each PCR were reamplified using a nested set of primers. For reverse transcription, a (-)sense primer was used hybridizing to the 5' noncoding region. For the two PCR amplification steps we used (+)sense primers hybridizing the 3' noncoding region and (-)sense primers hybridizing to the 5' noncoding region. After extraction with phenol/chloroform and ethanol precipitation, PCR products were digested with *NcoI* (incorporated in the (+)sense primer used in the nested PCR) and *EagI* (nucleotide 81 in the sequence of SEQ ID No. 1), and ligated into the *NcoI-EagI* sites of pUC21 (Vieira and Messing. 1991. Gene 100: 189-194).

All modification and cloning procedures used in Example 1 were carried out essentially as described (Sambrook et al. 1989. Molecular cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). Restriction enzymes and DNA modifying enzymes were commercially purchased and used as described by the suppliers. Plasmids were transformed and maintained in *Escherichia coli* strain DH5α (Hanahan. 1985. *in* DNA cloning 1: 109-135).

Sequencing of cDNA clones.

Plasmid DNA used for sequencing was extracted and purified ither by alkaline

lysis and LiCl precipitation, or by CsCl centrifugation (Sambrook t al. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). The T7 polymerase based sequencing kit (Pharmacia) was used for direct double stranded sequencing of plasmid DNA. In addition to the SP6, T7, and universal pUC/M13 forward and reverse primers, oligonucleotide primers were used based on the sequence of CSFV strain Brescia (Moormann *et al.* 1990. Virology 177: 184–198). Primers were synthesised with a Cyclone DNA synthesizer (New Brunswick Scientific) or with a 392 DNA/RNA synthesizer (Applied Biosystems). Sequence reactions were analysed on a 6% acrylamide gel containing 8 M urea. Sequence data were analysed with a Compaq 386 computer using Speedreader hardware, and PCgene software (Intelligenetics Inc, Applied Imaging Corp., Geneva, Switzerland) and with an Apple Macintosh computer using the program MacMolly*tetra*.

Considering the possibility of sequence errors or differences caused by Taq polymerase or heterogeneity of C-strain RNA, the entire genomic sequence of cDNA clones of the C-strain was determined by sequencing a minimum of two cDNA clones, obtained after independent PCR reactions. If differences were observed between the nucleotide sequences of two clones of a particular region, the consensus nucleotide sequence of that region was determined by sequencing a third cDNA clone obtained after another independent PCR reaction (Figure 1A).

Example 2

Generation of infectious transcripts of a full-length DNA copy of the genome of the C-strain.

Construction of cDNA clone pPRKflc-113. A full-length DNA copy of the genomic RNA of the C-strain was composed according to the scheme depicted in Figure 3. First, two subclones, one (pPRc64) containing the cDNA sequence of the 5' half of the genome (nucleotides 1-5,560), and the other (pPRc111) containing the cDNA sequence of the 3' half of the genome (nucleotides 5,463-12,311) were constructed. Initially construction of the full-length cDNA clone was tried in pGEM4z-blue. However, this approach failed because of instability of the full-length insert in this vector. To increase stability of the clones, inserts of the 5' and 3' half clones were recloned in a derivative of the low copy number vector pOK12 (Vieira and Messing. 1991. Gene 100: 189–194), resulting in pPRc108 and pPRc123, respectively. To this end pOK12 was modified by deleting most of the restriction sites of the multiple cloning site (MCS), and th T7 promoter sequence. Th resulting vector, pPRK, which was used for all further full-length cloning, still

contains unique *Spe*I, *Not*I, *Eag*I, *Bam*HI, *Eco*RV, *Eco*RI, and *Xba*I sites in the MCS.

In detail, the construction of full-length clone pPRKflc-113 pr ceeded as follows (Figure 3). Inserts of plasmids pPRc45 and pPRc46 were joined at the *Hpa*I site, located at nucleotide position 1249 in the sequence of the C-strain (SEQ ID No. 1), resulting in plasmid pPRc49. The insert of pPRc49 was subsequently joined with the insert of pPRc44 at the *Nsi*I site located at nucleotide position 3241 (SEQ ID No. 1), resulting in pPRc63. The 5' half clone pPRc64 (nucleotide 1 to 5560, SEQ ID No. 1) was constructed by joining the insert of pPRc63 with an amplified (PCR) cDNA fragment of the ultimate 5' region of the genomic RNA of the C-strain as follows. A 5' end (+)sense primer was synthesised containing an *Eco*RI and a *Sal*I site followed by the T7 RNA polymerase promoter sequence and the first 23 nucleotides of the genomic RNA of the C-strain. This primer and a (-) sense primer of the second round of cloning were used to amplify a cDNA fragment that was digested with *Eco*RI and *Xho*I cloned into *Eco*RI-*Xho*I (nucleotide 216 in SEQ ID No. 1) digested pPRc63. Finally, the insert of pPRc64 was recloned into *Eco*RI-*Xba*I digested pPRK resulting in pPRc108.

The 3' half clone pPRc111 (nucleotide 5,463 to 12,311, SEQ ID No. 1) was constructed by joining 4 second round clones (pPRc67, 53, 58, and 55) and one first round clone (pPRc14). The inserts of pPRc67 and pPRc53 were joined at the *Nhe*I site located at nucleotide position 7,778, resulting in pPRc71. The inserts of pPRc55 and pPRc58 were joined at the *Apa*I site located at nucleotide position 10,387, resulting in pPRc65. The inserts of pPRc65 and pPRc14 were subsequently joined at the *Afl*II site at nucleotide position 11,717, resulting in pPRc73. The insert of pPRc73 was joined with the insert of pPRc71 at the *Pst*I site located at nucleotide position 8,675, resulting in pPRc79. Then, the insert of pPRc79, which contains the complete 3' terminal sequence of the cDNA of the C-strain, was modified such that an *Srf*I site was introduced which after digestion generated the exact 3' end of the C-strain cDNA sequence (for exact run-off transcription at the 3'end). To achieve this, a 3' end (-)sense primer was synthesised containing an *Srf*I and an *Xba*I site and 18 nucleotides complementary to the 3' terminal sequence of the genomic RNA of the C-strain. This primer and a (+)sense primer of the first round of cloning were used to amplify a cDNA fragment. This fragment was digested with *Spe*I (nucleotide position 11,866, SEQ ID No. 1) and *Xba*I and cloned into *Spe*I-*Xba*I digested pPRc79, resulting in pPRc111.

Full-length cDNA clone pPRKflc-113, finally, was constructed by inserting the C-strain specific $NcoI^{5532}$-$XbaI^{mcs}$ fragm nt of pPRc111 into $NcoI^{5532}$-$XbaI^{mcs}$ digested pPRc108.

Construction of full-length clone pPRKflc-133.

Full-length cDNA clone pPRKflc-113 still had, besides silent nucleotide mutations, 5 point mutations leading to amino acid changes compared to the amino acid sequence determined from the sequence of at least two first round cDNA clones. These 5 point mutations in pPRKflc-113 were changed to the predominant sequence (2 out of 3) through exchange of affected DNA fragments with corresponding DNA fragments containing the predominant sequence.

The 5' half cDNA clone pPRc108, with a point mutation at nucleotide position 4,516, was changed by replacing the *Sca*I^{3413}-*Nco*I^{5532} fragment of pPRc108 with that of pPRc124. Clone pPRc124 was made by exchanging the *Pvu*II^{4485}-*Nhe*I^{5065} fragment of pPRc44 by the corresponding fragment of pPRc32 (compare Figure 1). The new 5' half cDNA clone was designated pPRc129.

For cloning purposes a 3' half clone was constructed by deleting the 5' part of the C-strain sequence of pPRKflc-113 from the *Sal*I site in the vector (compare Figure 3) up to the *Hpa*I site at nucleotide position 5,509 (SEQ ID No. 1), resulting in pPRc123. In pPRc123 mutations at nucleotide positions 8,526, 9,002, 10,055, and 10,205 had to be changed. The mutation at position 8,526 was restored in two steps. First, the *Apa*I^{8,506}-*Pst*I^{8,675} fragment of pPRc53 was exchanged with that of pPRc90, resulting in pPRc125. Second, the *Nhe*I^{7,378}-*Pst*I^{8,675} fragment of pPRc123 was exchanged with that of pPRc125, resulting in pPRc127.

To be able to restore the 3 mutations at positions 9,002, 10,055, and 10,205, we first modified pPRc58 such that the *Fsp*I site in the vector was deleted. To this end the *Eco*RI^{mcs}-*Nde*I fragment of pPRc58 was deleted (*Nde*I cuts in pGEM4z-blue), resulting in pPRc126. Plasmid pPRc126 was used for restoring the mutations at positions 10,055 and 10,205 by replacing its *Sac*I^{9,975}-*Apa*I^{10,387} fragment with the corresponding fragment of pPRc96, resulting in pPRc128. The mutation at position 9002 was restored by replacing the AatII-*Fsp*I^{9016} (*Aat*II cuts in pGEM4z-blue) of pPRc128 with the *Aat*II-*Fsp*I^{9,016} fragment of pPRc90, resulting in pPRc130. Finally, the *Pst*I^{8,675}-*Apa*I^{10,387} fragment of pPRc127 was replaced with the corresponding fragment of pPRc130, resulting in plasmid pPRc132. All subcloning steps in which single mutations were changed were verified by sequencing.

Full-length clone pPRKflc-133 was constructed by inserting the *Nco*I^{5,532}-*Xba*I^{mcs} fragment of pPRc132 into *Nco*I^{5,532}-*Xba*I^{mcs} digested pPRc129.

Construction of a hybrid full-length clone pPRKflc-h6.

Antigenically different but viable C-strain mutants can be made from pPRKflc-133, by exchanging part of the E1 gene of this construct with that of CSFV strain Brescia. To this end, the *Nhe*I^{2,443}-*Afl*II^{2,999} fragment of pPRc129 was replaced with the corresponding fragment of pPEh6 (van Rijn *et al.*, 1992), resulting in the 5' half hybrid clone pPRc139. Hybrid full-length clone pPRKflc-h6 was constructed by inserting the *Nco*I^{5,532}-*Xba*I^{mcs} fragment of pPRc132 into pPRc139. This clone now contained the antigenic region of E1 of CSFV-strain Brescia including a unique *BglII* site.

All modification and cloning procedures used in Example 2 were carried out essentially as described (Sambrook et al. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). Restriction enzymes and DNA modifying enzymes were commercially purchased and used as described by the suppliers. Plasmids were transformed and maintained in *Escherichia coli* strain DH5α (Hanahan. 1985. *in* DNA cloning 1: 109-135).

In vitro RNA transcription

Plasmid DNA used for *in vitro* RNA transcription was purified using Qiagen columns (Westburg), according to manufacturers conditions. After linearisation with *Xba*I or *Srf*I, plasmid DNA was extracted with phenol and chloroform, precipitated with ethanol, vacuum dried and dissolved in an appropriate volume of RNase-free water.

One μg of linearised plasmid DNA was used as template for *in vitro* transcription. RNA was synthesised at 37°C for 1 hr in 100 μl reaction mixtures containing 40 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 2 mM Spermidine, 10 mM DTT, 100 U rRNasin (Promega), 0.5 mM each of ATP, GTP, CTP, UTP and 170 Units of T7 RNA polymerase (Pharmacia). Template DNA was removed by digestion with RNase-free DNaseI (Pharmacia) for 15 min. at 37°C, followed by extraction with phenol and chloroform, and ethanol precipitation. The RNA was dissolved in 20 μl RNase-free H_2O, and quantitated by UV measurement at 260 nm.

RNA transfection

The RNA transfection mix was composed by gently mixing 50 μl of a lipofectine (Gibco BRL) dilution (10 μg lipofectine in RNase-free H_2O) and 50 μl of an RNA solution (1 μg RNA in RNase-free H_2O), and incubation of this mix at room temperature for 15 minutes. Subconfluent monolayers of SK6-cells in ϕ 35 mm, 6-well tissue culture plates (Greiner) were used for RNA transfection. The cells were washed twice with Dulbecco's

modified Eagles medium (DMEM). Then 1 ml of DMEM was added to the cells, followed by the RNA transfection mix. After incubation for 16 hrs at 37°C, th medium was replaced by 2 ml DMEM supplemented with 5% FBS. Incubation was continued for another 3 days at 37°C. Then cells were immunostained with CSFV specific monoclonal antibodies (Mabs) by the immunoperoxidase monolayer assay (IPMA) as described by Wensvoort *et al.* (Vet. Microbiol. 1986. 12: 101-108).

Characterisation of recombinant C-strain viruses.

The supernatants of transfected cells were brought on confluent monolayers of SK6-cells in wells of ϕ 35 mm, and incubated for 5 days at 37°C. Cells of the transfected monolayers were trypsinised and diluted 7.5 times with DMEM and grown for another 7 days at 37°C in 75 cm^2 flasks (Costar). Hereafter, virus stocks were prepared by freeze-thawing the cells twice, clarifying cell suspensions by centrifugation at 5,000 x *g* for 10 min. at 4°C, and harvesting of the supernatants.

Virus was characterised by IPMA, and by restriction analysis of RT-PCR amplified viral fragments. After infection of SK6-cells with viruses FLc-h6 and Flc-133, monolayers were incubated for 4 days at 37°C. Subsequently, monolayers were immunostained using Mabs directed against conserved (Mab b3, domain A) and non-conserved (Mabs b5 and b6, domains B+C) epitopes on E1 of Brescia, and with Mabs specific for the C-strain and directed against E1 (Mab c2) or E2 (Mab c5) (Wensvoort, G. 1989. *In* Thesis, pp 99-113, Utrecht, The Netherlands). Monolayers of SK6-cells infected with native Brescia virus or native C-strain virus were controls in this assay. The results are presented in Table 1, and are as expected. Briefly, Mab b3 recognizes an epitope on E1 conserved among CSFV strains, and therefore recognizes all strains in Table 1. Mabs b5 and b6 do not recognize E1 of the C-strain and thus only react with strains Brescia and Flc-h6. In contrast, Mabc2 does not recognize E1 of strain Brescia, and thus only reacts with strains C and FLc-133. Finally, Mab c5 does not recognize E2 of strain Brescia, and therefore reacts with all viruses in Table 1 except strain Brescia.

The genomic RNA of virus FLc-h6 should contain a unique *Bgl*I site, which is located in the E1 gene (see above). To check for the presence of this site, cytoplasmic RNA was isolated from SK6-cells infected with recombinant virus FLc-h6, or infected with FLc-133, PCR-amplified as described above, using primers described by Van Rijn *et al.*, 1993. J. Gen. Virol. 74:2053-2060), and digested with *Bgl*I. Indeed, the amplified fragment of 1,091 basepairs of FLc-h6 was cut by *Bgl*I, resulting in fragments of 590 and 501 basepairs, whereas the amplified fragment of FLc-133 remained intact.

Example 3

Immunisati n of pigs with deletion mutants of E1.

Construction and expression of deletion mutants of E1 of CSFV strain Brescia.

It was previously shown that TMR-less E1 of CSFV strain Brescia, expressed by insect cells, induces a protective immune response in pigs against CSF (Hulst *et al.*, 1993. J. Virol. 67: 5435-5442). Two distinct antigenic units, A and B+C, in the N-terminal half of E1, which induce neutralizing antibodies against CSFV, were also defined (Wensvoort. 1989. J. Gen. Virol. 70: 2865-2876; Van Rijn *et al.* 1992. Vet. Microbiol. 33: 221-230; Van Rijn *et al.* 1993. J. Gen. Virol. 74: 2053-2060). Moreover, neutralizing antibodies directed against domain A and domains B+C act synergistically in neutralizing CSFV (Wensvoort. 1989. J. Gen. Virol. 70: 2865-2876). To evaluate the immunogenicity of mutant E1s with a deletion of domains B+C or with a deletion of domain A, relevant constructs in a baculovirus vector were made, and expressed mutant proteins were tested in pigs.

Baculoviruses expressing mutant E1s were constructed via overlap recombination of wild type AcNPV (*Autographa californica* nuclear polyhedrosis virus) DNA and DNA of transfer vector pAcMo8 containing the sequence encoding a particular mutant E1. Transfer vector pAcMo8 was derived from pAcAs3 (Vlak et al., 1990. Virology 179: 312-320) by inserting a T directly 5' of the first base (G) of the unique *Bam*H1 site of the latter vector. In this way an ATG start codon was generated overlapping the first G of the *Bam*HI site. Messenger RNA is transcribed from heterologous sequences inserted into the *Bam*HI site by the AcNPV p10 promoter.

The sequences encoding mutant E1s were derived from the E1 insert of pPRb2 (Van Rijn *et al.*, 1992. Vet. Microbiol. 33: 221-230) via PCR amplification. To this end two primers were constructed which contained a *Bam*HI site in their sequence. The 5' end (+ sense) primer has the sequence 5'-AGA **TTG GAT CCT** AAA GTA TTA AGA GGA CAG GT-3' (SEQ ID No. 2). The underlined sequence in this primer is identical to nucleotides 2362-2381 in the sequence of strain Brescia (Moormann *et al.*, 1990. Virology 177: 184-198), bold letters indicate the *Bam*HI site. The 3' end (- sense) primer contains a stop-codon adjacent to the BamH1 site. It has the sequence 5'-TA GTC **GGA TCC** *TTA* GAA TTC TGC GAA GTA ATC TGA-3' (SEQ ID No. 3). The underlined sequence in this primer is complementary to nucleotides 3433-3453 in the sequence of strain Brescia (Moormann *et al.*, 1990. Virology 177: 184-198); bold letters indicate the *Bam*HI site, and letters in italics indicate the stop-codon.

Amplified sequences were cl ned into the *Bam*HI site of pAcMo8 and checked

for a corr ct orientation in the vector by restriction enzyme analysis. The corr ct transfer vector was designated pPAb11. Overlap recombination between AcNPV DNA and DNA of pPAb11, and selection and purification of a baculovirus vector expressing E1 was performed as described (Hulst *et al.*, 1993. J. Virol. 67: 5435-5442). Further characterisation of E1 in radioimmunoprecipitation assays, and with E1 specific Mabs was also described by Hulst *et al.* (J. Virol., 1993. 67: 5435-5442). The resulting recombinant baculovirus expresses wild-type Brescia E1 without a TMR (compare 2nd bar from top in Figure 3). This TMR-less E1 is secreted from the cells (Hulst *et al.*, 1993. J. Virol. 67: 5435-5442).

Deletion of the region encoding domains B+C from the E1 gene of pPAb11 was achieved by exchanging the *Nhe*I-*Bgl*II fragment of this construct with the corresponding fragment of pPEh14 (Van Rijn *et al.*, 1993. J. Gen. Virol. 74: 2053-2060). The resulting transfer vector was designated pPAb16. It contains a deletion in the E1 gene running from codon 693 to 746. Similarly, the region encoding domain A was deleted from pPAb11 by exchanging the *Nhe*I-*Bgl*II fragment of pPAb11 with the corresponding fragment of pPEh18 (Van Rijn *et al.*, 1993. J. Gen. Virol. 74: 2053-2060), resulting in transfer vector pPAb12. pPAb12 contains a deletion in the E1 gene running from codon 800 to 864.

Recombinant baculoviruses expressing the deleted E1s were constructed, selected, and characterised with regard to their E1 expression products, as described above.

Immunisation and challenge exposure of pigs.

Groups of four (or two) specific-pathogen-free (SPF), 6 to 8 week old pigs were vaccinated intramuscularly on day 0 with 1 ml of a double water-oil emulsion containing 4 μg (mutant) E1, and revaccinated on day 28 with 1 ml of a double water-oil emulsion containing 15 μg (mutant) E1 (Table 2). The construction of mutant E1 containing a deletion in domain A, or a deletion in domain B/C, and of wild type E1 is described above and specified by the constructs depicted in Figure 5. For the first vaccination on day 0, supernatant of insect cells infected with the appropriate recombinant baculoviruses was used. The amount of E1 in the supernatant was calibrated as described before (Hulst *et al.*, 1993. J. Virol. 67: 5435-5442). For revaccination on day 28, E1 was immunoaffinity-purified from the supernatant of the infected insect cells (Hulst *et al.*, 1993. ibid). Pigs of all vaccinated groups, and an unvaccinated control gr up of two SPF animals, were challenged intranasally with 100 LD_{50} of CSFV strain Brescia 456610

(Terpstra and Wensvoort. 1988. Vet. Microbiol. 16: 123–128). This challenge dose leads to acute disease in unprotected pigs characterised by high fever and thrombocytopenia starting at days 3 to 5 and to death at days 7 to 11. Heparinised (EDTA) blood samples were taken on days 40, 42, 45, 47, 49 , 51, 53, and 56 after vaccination, and analysed for thrombocytes and CSFV virus as described (Hulst *et al.*, 1993. ibid). Serum blood samples were taken on days 0, 21, 28, 42, and 56 and tested in the CTB–ELISA (Wensvoort et al., 1988. Vet. Microbiol. 17: 129–140) and in the neutralizing peroxidase–linked assay (NPLA, Terpstra et al. 1984. Vet. Microbiol. 9: 113–120), to detect (neutralizing) antibodies against CSFV. Test results in the CTB–ELISA are expressed as the percentage inhibition of a standard signal; <30% inhibition is negative, 30–50% inhibition is doubtful, >50% inhibition is positive. NPLA titers are expressed as the reciprocal of the serum dilution that neutralised 100 $TCID_{50}$ of strain Brescia in 50% of the replicate cultures.

All animals were observed daily for signs of disease, and body temperatures were measured. Clinical signs of disease were: Fever, anorexia,leukopenia, thrombocytopenia, and paralysis.

Example 4

Development of a CTB–ELISA (CTB–DIF) for CSFV, based on one monoclonal antibody.

Description of the diagnostic test.

This example describes a CTB–ELISA (CTB–DIF), which is a modification of the existing CTB–ELISA (Wensvoort et al., 1988. Vet. Microbiol. 17: 129–140) for the detection of CSFV specific antibodies.

The CTB–DIF is based on the finding that SF21 cells infected with a recombinant baculovirus expressing E1–TMR, efficiently secrete dimerised E1 into the medium. This dimerised secreted E1 was detected when media of cells infected with the above baculovirus was analysed on western blot after electrophoresis in SDS–PAGE under non–reduced conditions. For E1 specific Mabs, two copies of an epitope are present on dimers of E1 (one on each monomer). Thus, in conjunction with the dimerised antigen a particular E1 specific Mab can be used as capture antibody, coated to the wall of a microtiter plate well, as well as detecting, horseradish peroxidase (HRPO) conjugated antibody.

Th CTB–DIF is shown to be useful in conjunction with an E1 subunit vaccine which has a deletion in domain A (see Fig. 5 for construct) and is shown to distinguish

between CSFV specific antibodies induced in pigs vaccinated with E1 with a deleted domain A, and CSFV specific antibodies induced in pigs infected with low-virulent CSFV strains Henken, Zoelen, Bergen, 331, and Cedipest (EP-A-351901).

Four SPF pigs, numbered 766, 786, 789, and 770, were vaccinated with mutant E1 containing a deletion in domain A, as described in example 3 (see also Table 2), and challenged with virulent CSFV strain Brescia on day 44 after vaccination. Sera taken on days 28, 42, and 56 after vaccination, were tested.

Sera against the low-virulent CSFV strains were also prepared in groups of four SPF pigs. Sera from pigs infected with strains Henken, Zoelen, Bergen, and 331 were tested at days 0, 21, 28 and 42 after infection. Sera from pigs vaccinated with the Cedipest vaccine were tested at days 0, 44, 72, and 170 after vaccination.

Three different serological tests were performed with the above sera. Test 1 is the neutralizing peroxidase-linked assay (NPLA) described by Terpstra et al. 1984. (Vet. Microbiol. 9: 113-120), to detect neutralizing antibodies against CSFV. Test 2 is the CTB-ELISA (Wensvoort et al., 1988. Vet. Microbiol. 17: 129-140), to routinely detect antibodies against CSFV.

The CTB-DIF uses Mab b3 (also known as CVI-HCV-39.5) (Wensvoort. 1989. J. Gen. Virol. 70:2865-2876.), which recognizes an epitope located in domain A1 of E1 of CSFV. The wells of an ELISA plate are coated with Mab b3 (dilution 1:2.000) (capture antibody). After the wells are washed, Mab b3, conjugated to (HRPO), (dilution 1:4.000) (detecting antibody), is added to the wells. Media of Sf21 cells infected with a baculovirus producing E1-TMR and containing dimerised E1 to a concentration of 20 µg/ml is diluted 1:500, and pre-incubated with the test serum (diluted 1:2.5). The serum-antigen mixture is then added to the conjugate in the wells of the coated ELISA plate. After incubation, the wells are washed again and the chromogen-substrate solution is added. If both the capture and conjugated Mab have bound to the antigen, the HRPO induces a chromogenic reaction, indicating that the test serum is negative for CSFV antibodies. If the epitope on the antigen is blocked by antibodies from the test serum, the HRPO-conjugate will be washed away and the wells will remain clear, indicating the test serum contains antibodies against CSFV, domain A1. The results with the three different serological tests are indicated in Table 3.

Sera of pigs vaccinated with E1 with a deletion in domain A, do react in the NPLA and CTB-ELISA, and not in the CTB-DIF, on day 42 after vaccination. After challenge with virulent CSFV strain Brescia sera of the same pigs react positively in all the 3 tests on day 56 after vaccination (day 12 after challenge), indicating that a booster

response has taken place after challenge. Starting on day 21 after infection, s ra from pigs vaccinated with strains Henken, Zoelen, Bergen, and 331 react positively in the NPLA, the CTB-ELISA, and the CTB-DIF. Starting on day 44 after vaccination, the same holds true for pigs vaccinated with the Cedipest vaccine strain.

Thus, the CTB-DIF exactly performs as desired, and is suited to accompany a CSFV marker vaccine with a mutated domain A of E1, such that antibodies directed against this mutated domain A do not compete with Mab b3 for the epitope of Mab b3.

The antigen used in the CTB-DIF is the dimerised TMR-less wild type Brescia E1 depicted in Figure 5. However, dimerised E1 synthesised by the "deletion domains B+C" construct of Figure 5 is also suitable as an antigen in the test.

Example 5

Comparison of CTB-ELISA's for CSFV based on E1 and E2.

Description of the diagnostic tests

This example describes a modification of the CTB-DIF of example 4, and a CTB-ELISA based on E2 of CSFV, and compares the sensitivity of these ELISAs with 3 other CTB-ELISAs detecting antibodies directed against E1 and the NPLA (Terpstra et al. 1984. Vet. Microbiol. 9: 113-120).

The CTB-DIF of example 4, called E1-Bac-DIF in Tables 4 to 8, uses intact TMR-less E1 synthesized in insect cells (SF21 cells) as an antigen. The modification of E1-Bac-DIF, called E1-Bac-dBC-DIF, uses TMR-less E1 synthesized in insect cells (SF 21 cells) with a deleted domain B + C (compare figure 5) as an antigen. As established on western blot, TMR-less E1 with deleted domains B + C is secreted from the cell as a dimer (results not shown). Test E1-bac-dBC-DIF is performed as follows. The wells of an ELISA plate are coated with Mab b3 (dilution 1:4,000) (capture antibody), 16 h at 37 °C, and washed. Medium containing dimerised antigen E1-dBC to a concentration of 20 µg/ml is diluted 1:50, and pre-incubated with the test serum (dilution 1:2.5) (0.5 h at 37 °C). The serum-antigen mixture is then added to the coated ELISA plate. After incubation, 1 h at 37 °C, the wells are washed and Mab b3, conjugated to HRPO (dilution 1:1,000) (detection antibody), is added. After incubation, 1 h at 37 °C, the wells are washed again and the chromogen-substrate solution is added. The chromogenic reaction is performed for 10 minutes at room temperature. The interpretation of the chromogenic reaction is the same as explained in example 4.

Other CTB-ELISAs detecting antibodies directed against E1 of CSFV described in Tables 4 to 8 are th E1-CSFV ELISA, using native E1 from CSFV infected cells as

antigen (Wensvoort et al., 1988. Vet. Microbiol. 17:129-140); the E1-Bac and E1-Bac-DIF ELISAs, use TMR-less E1 synthesized in insect cells as antigen. The E1-CSFV and E1-Bac ELISAs use CSFV Mabs b3 and b8 (Wensvoort 1989. J. Gen. Virol. 70: 2,865-2,876) as capture and detection antibody, respectively, whereas the E1-Bac-DIF ELISA uses only Mab b3 as both capture and detection antibody. The E1-CSFV ELISA is performed exactly as described by Wensvoort et al., 1988. (Vet. Microbiol. 17:129-140). The E1-Bac, and E1-Bac-DIF ELISAs are performed as described above for the E1-Bac-dBC-DIF with the following modifications. In the E1-Bac ELISA the antigen used is a 1:400 dilution of dimerized E1 present in the medium of SF21 cells, infected with the relevant E1 baculovirus construct (compare figure 5), at a concentration of 20 µg/ml. Mab b8 which is conjugated to HRPO is the detection antibody in this ELISA, and is used at a dilution of 1:1000. The E1-Bac-DIF ELISA uses the same antigen as the E1-Bac ELISA but at a dilution of 1:200. HRPO conjugated Mab b3 is used as detection antibody in this ELISA at a dilution of 1:1,000.

The E2-Bac ELISA uses CSFV E2 antigen synthesized in SF21 cells infected with the Bac CE2 construct (Hulst et al., 1994. Virology 200: 558-565). Because E2 is not secreted from the infected insect cells, the lysate of these cells is used. Like E1, most of E2 is found as dimerized molecules when lysates of infected cells are analyzed under non-denaturing conditions in SDS-PAGE gels (results not shown). The CTB-ELISA developed on the basis of this E2 antigen performs optimally in conjunction with Mabs C5 and C12 (Wensvoort, G. 1989. *In* Thesis, pp99-113, Utrecht). However, also E2 in conjunction with only Mab C5 or Mab C12 may be used. In a competition assay Mabs C5 and C12 inhibit each other with regard to binding to E2. This indicates that these Mabs recognize the same, or overlapping epitopes on E2 (results not shown). The E2-Bac ELISA is performed as follows. Mab C12 is diluted 1:1,000, and coated to the wells of an ELISA plate (16 h at 37 °C). Hereafter, wells are washed. Lysates of SF21 cells infected with Bac CE2, diluted 1:1,250, are preincubated with the test serum (1:1) for 0.5 h at 37 °C. The serum-antigen mixture is then added to the wells of the coated plates and incubated for 1h at 37 °C. Subsequently plates are washed and incubated with Mab C5 conjugated to HRPO (dilution 1:2,000). After 1h at 37 °C plates are washed again and the chromogen-substrate solution is added. The chromogenic reaction is performed for 10 minutes at room temperature. The interpretation of the chromogenic reaction is the same as explained in example 4. All above described dilutions are performed in NPLA buffer + 4% PS (Terpstra et al., Vet. Microbiol. 9: 113-120).

Table 4 shows the results of the analysis of sera of 3 SPF pigs vaccinated with the Cedipest vaccine with the above described CTB-ELISAs and the NPLA. Sera were analyzed at days 0, 16, 23, 30, 37, 44, 50, 72, 113, 141, and 170 after vaccination. Tables 5 to 8 show the results of the analysis with the above described CTB-ELISAs and the NPLA of sera of groups of 5 SPF pigs infected with the low-virulent CSFV strains 331, Bergen, Henken, and Zoelen, respectively. Sera were analyzed at days 0, 10, 14, 17, 24, 28, 35, and 42 after infection. Starting at day 16 after vaccination, sera from pigs vaccinated with the Cedipest strain react in each of the 5 CTB-ELISAs as well as in the NPLA. At this time point the sensitivity of the E2-Bac ELISA and the E1-Bac-dBC-DIF is as good, if not better, than that of the other 3 CTB-ELISAs. From day 37 after vaccination up till day 170, all sera react consistently (positive) in the 5 CTB-ELISAs as well as the NPLA. Sera of pigs infected with the low-virulent CSFV strains also react in all 5 CTB-ELISAs as well as in the NPLA. With an occasional exception consistency in the reaction of the sera in the 5 CTB-ELISAs and the NPLA is observed from day 21 after infection up till day 42. More sera of animals infected with low virulent strains need to be analyzed to be able to conclude whether there are significant differences between the sensitivity of the 5 CTB-ELISAs early after infection (up till day 17).

It can be concluded that the E2-Bac ELISA and the E1-Bac-CTB-DIF ELISA both perform as desired. Therefore the E2-Bac ELISA is suitable to accompany a CSFV marker vaccine (eg. subunit E1, whether mutated or not, a C-strain marker vaccine modified in the E2 region) which does not induce antibodies that compete with the Mabs in this ELISA. The E1-Bac-dBC-DIF ELISA is as suitable as the E1-Bac-DIF ELISA (CTB-DIF ELISA of example 4) to accompany a CSFV marker vaccine with a mutated domain A of E1, such that antibodies directed against this mutated domain A do not compete with Mab b3 for the epitope of Mab b3.

Description of the figures

Figure 1.

Schematic representation of the cDNA clones used to determine the nucleotide sequence of the C-strain. Figure 1A indicates the first round cDNA clones (see text). cDNA clones with numbers 32, 90, and 96 were used to change pPRKflc-113 into pPRKflc-133 (see example 2). Clone 14 was the only first round cDNA clone used for construction of pPRKflc-113 (see Figure 3). Figur 1B indicates second round cDNA clones (see text). The numbered second round cDNA clones were used to construct

pPRKflc-113 (see SEQ ID No. 1). Positions of the cDNA with respect to the nucleotide sequence of the genome of the C-strain are indicated by the scale bar (in kilobases) at the bottom of the figure. A schematic representation of the currently identified genes of CSFV, and their organisation in the CSFV genome is indicated at the top of the figure.

There is no consensus yet among workers in the field about the nomenclature of pestivirus proteins. The E2 protein as described here is also called gp42 (Tamura et al. 1993. Virology 193: 1-10), gp44/48 (Thiel et al. 1991. J. Virol. 65: 4705-4712) or E0 (Rümenapf et al. 1993. J. Virol. 67: 3288-3294). The E3 protein is also called gp25 (Tamura et al. 1993. Virology 193: 1-10), gp33 (Thiel et al. 1991. J. Virol. 65: 4705-4712) or E1 (Rümenapf et al. 1993. J. Virol. 67: 3288-3294). The E1 protein of this invention is also called gp53 (Tamura et al. 1993. Virology 193: 1-10), gp55 (Thiel et al. 1991. J. Virol. 65: 4705-4712), gp51-54 (Moormann et al. 1990. Virology 177: 184-198) and E2 (Rümenapf et al. 1993. J. Virol. 67: 3288-3294). The N-terminal autoprotease N^{pro} of CSFV (p20 of BVDV, Wiskerchen et al. 1991. J. Virol. 64: 4508-4514), also called p23, was identified by Thiel et al. 1991. (J. Virol. 65: 4705-4712). Cleavage of the recognition sequence, which is conserved among pestiviruses, of this protease results in the N-terminus of C (Stark et al. 1993. J. Virol. 67: 7088-7095).

Figure 2.

Alignment of the nucleotide sequences of the 5' (A) and 3' (B) non-coding regions of CSFV strains Brescia, Alfort, and C. Except for the first 12 nucleotides, the 5' non-coding sequence of strain Brescia has been described by Moormann et al., 1990. Virology 177: 184-198. The first 12 nucleotides of the 5' non-coding region of strain Brescia have not been published before. Like the ultimate 5' and 3' sequences of the genome of the C-strain, they were determined with the 3'-5' RNA ligation method described in Example 1 of this patent application. Except for the first 9 nucleotides, the 5' non-coding sequence of strain Alfort has been described by Meyers et al., 1989. Virology 171: 555-567. The first 9 nucleotides of the genome of strain Alfort were published by Meyers in a Thesis entitled: "Virus der Klassischen Schweinepest: Genomanalyse und Vergleich mit dem Virus der Bovinen Viralen Diarrhöe". 1990. Tübingen, Germany. The sequences of the 3' non-coding regions of strains Brescia and Alfort have been described by Moormann et al., 1990. Virology 177: 184-198 and Meyers et al., 1989. Virology 171: 555-567, respectively. The ATG start codon and the TGA stopcodon of the large ORF (compare SEQ ID No. 1), ar underlined.

Figure 3.

Construction scheme of full-length cDNA clone pPRKflc-113. Clone numbers have been explained in the legend of Figure 1. Fusion sites of inserts of clones are indicated by vertical lines. The sites corresponding with these lines are indicated at the bottom of the figure. Underlined clone numbers indicate cDNA clones having pOK12 (Vieira and Messing. 1991. Gene **100:** 189-194) derived vector sequences (see Figure 4). The 5' and 3' ends of pPRKflc-113 were tailor made via PCR amplification of cDNA fragments (see text Example 2). The amplified fragments are indicated with PCR. The scale bar at the bottom of the figure, and the schematic representation of the genome organisation of CSFV, have been described in the legend of Figure 1.

Figure 4.

Schematic representation of the vector sequences and full-length cDNA inserts in clones pPRKflc-113, pPRKflc-133, and pPRKflc-h6. The construction of vector pPRK, a derivative of pOK12 (Vieira and Messing. 1991. Gene **100:** 189-194), has been described in Example 2. Kan^R, kanamycin resistance gene; ORI, origin of replication; 'I, gene encoding repressor of B-galactosidase gene; PO, promoter/operator region of β-galactosidase gene; lacZ, part of the β-galactosidase gene encoding the α subunit of β-galactosidase. Several restriction sites of the vector, and the sequences directly flanking the full-length inserts in the vector, are indicated. Relevant sites have been described in the text of Example 2. The lollypops and numbers in pPRKflc-113 correspond to the nucleotides of the five codons which were changed in this construct, resulting in pPRKflc-133. The latter construct has the sequence as indicated in SEQ ID No. 1.

The black box in pPRKflc-h6 indicates the region of E1 of pPRKflc-133 that was exchanged with the corresponding region of strain Brescia. Whether transcripts derived from a particular full-length construct are infectious (+) or not (-) is indicated to the right of the construct. T7, T7 promoter sequence. Inserts of full-length constructs are indicated in relation to a scale bar (in kilobases) representing the nucleotide sequence of the C-strain as indicated in SEQ ID No. 1.

Figure 5.

Schematic representation of mutant E1 proteins expressed in insect cells with a baculovirus vector. All E1 proteins are ncoded by the nucleotide sequence of strain Brescia (Moormann et al., 1990. Virology 177: 184-198), and start at their N-terminus

with the Lys at codon position 668 in the large ORF of this sequence. The C-terminus of native E1 is the Leu at codon position 1,063 in the larg ORF, whereas the C-termini of the three other E1 proteins are located at amino acid position 1,031. The dotted boxes in the bars represent the N-terminal signal sequence, running from amino acid residues 668 to 689, the internal hydrophobic sequence, running from amino acid residues 806 to 826, and the C-terminal transmembrane region (TMR), located in the region running from amino acid residues 1,032 to 1,063, of E1. The deleted amino acid sequences in mutant E1s with a deleted B+C or A domain are indicated by interruptions in the bars representing these proteins. The location of these deletions in relation to the amino acid sequence of E1 can be determined from the scale bar at the bottom of the figure. The scale bar indicates the location of E1 in the amino acid sequence encoded by the large ORF of strain Brescia.

TABLE 1. Characterisation of recombinant C-strain viruses

	Mabs specific for CSFV			
	directed against E1			directed against E2
virus	conserved epitopes	Brescia specific epitopes	"C" specific epitopes	"C" specific epitopes
"C"	+	-	+	+
Brescia	+	+	-	-
FLc-133	+	-	+	+
FLc-h6	+	+	-	+

TABLE 2. Vaccination of pigs with deletion mutants of E1

		% inhibition in the CTB-ELISA on days:					Neutralizing antibody titer on days:					Results of CSFV challenge:		
Pig no.	Construct used	0	21	28	42	56	0	21	28	42	56	Disease	Viremia	Death
766	Deletion	0	12	0	78	99	<12.5	<25	<25	3,200	>3,200	- - - - -	-	-
768	Domain	0	0	0	74	99	<12.5	<25	<25	2,400	>3,200	- - - - -	-	-
769	A	0	16	0	99	99	<12.5	<25	<25	2,400	>3,200	- - - - -	-	-
770		0	14	0	83	99	<12.5	<25	<25	400	>3,200		-	-
771	Deletion	0	36	25	100	99	<12.5	<25	<25	300	>3,200	- - - - -	-	-
772	Domain	0	25	29	100	99	<12.5	<25	<25	1,200	>3,200	- - - - -	-	-
773	B+C	0	7	0	100	99	<12.5	<25	<25	200	150	- - - - -	-	-
774		0	49	52	99	99	<12.5	<25	<25	300	>3,200	- - - - -	-	-
792	Wild type	0	35	31	98	100	<12.5	2,000	1,200	>3,200	>3,200	+ - - - -	-	-
794	BresciaE1	0	48	40	98	100	<12.5	<25	<25	1,600	>3,200	+ - - - -	-	-
775	None	0	2	0	0		<12.5	<25	<25	<25		+ + + + +	+	+
795		0	0	0	0		<12.5	<25	<25	<25		+ + + + +	+	+

Groups of four (or two) SPF pigs were inoculated intramuscularly on day 0 with 4 µg and on day 28 with 15 µg modified E1 protein. On day 42, the pigs were challenged intranasally with 100 LD50 of CSF strain Brescia 456610. All animals were observed daily for signs of disease. Blood samples were taken at days 0, 21, 28, 42, and 56 and tested in the CTB-ELISA and the neutralisation test for the detection of antibodies against CSFV (Terpstra et al., 1984. Vet. Microbiol. 9: 113-120). Clinical signs of disease were : Fever, anorexia, leukopenia, thrombocytopenia, and paralysis and the presence or absence of these signs is indicated in that order by a + or - in the table listed under disease.

TABLE 3. Differential diagnostic ELISA test for CSFV

Serum	DPV or DPI[a]	NPLA[b]	CTB-ELISA[c]	CTB-DIF[c]
766	28	<25	9	27
768	28	<25	0	27
769	28	<25	17	0
770	28	<25	11	0
766	42	3200	81	0
768	42	2400	52	0
769	42	2400	99	26
770	42	400	65	0
766	56	>3200	99	65
768	56	>3200	100	104
769	56	>3200	101	105
770	56	>3200	101	104
Henken	0	<12.5	5	18
Henken	21	50	76	47
Henken	28	75	92	88
Henken	42	300	100	102
Zoelen	0	<12.5	0	0
Zoelen	17	37.5	85	71
Zoelen	21	150	90	80
Zoelen	42	400	100	108
Bergen	0	<12.5	1	49
Bergen	21	25	96	100
Bergen	28	100	99	95
Bergen	42	300	100	103
331	0	18.75	4	15
331	21	100	92	90
331	28	300	99	99
331	42	300	100	105
Cedipest	0	<12.5	0	19
Cedipest	44	75	85	93
Cedipest	72	50	89	102
Cedipest	170	150	98	108

[a]) DPV: days post vaccination; DPI: days post infection

[b]) NPLA titers are expressed as the reciprocal of the serum dilution neutralizing 100 $TCID_{50}$ of HCV strain Brescia in 50% of the replicate cultures (Terpstra et al. 1984. Vet Microbiol. 9:113-120).

[c]) Complex trapping blocking-ELISA, CTB-ELISA, or differential CTB-ELISA, CTB-DIF. Test results are expressed as the percentage inhibition of a standard signal; <30% inhibition is negative, 30-50% inhibition is doubtful, >50% inhibition is positive.

TABLE 4. Comparison of CTB-ELISA's with CSFV strain Cedipest sera

strain	pig	DPV[a] or DPI	NPLA[b]	CTB-ELISA[c] E1 CSFV	E1-Bac	E1-Bac DIF	E1-Bac dBC-DIF	E2-Bac
Cedi-pest	1	0	<12.5	21	ND	ND	ND	0
	2	0	<12.5	28	8	0	0	0
	3	0	<12.5	25	0	0	0	0
	1	16	25	60	0	56	62	79
	2	16	25	66	51	26	76	78
	3	16	19	11	15	0	11	62
	1	23	25	54	66	60	68	79
	2	23	50	81	57	54	75	74
	3	23	25	25	37	32	54	74
	1	30	50	76	87	80	81	75
	2	30	75	87	ND	ND	ND	ND
	3	30	19	60	40	28	57	82
	1	37	50	82	90	80	87	85
	2	37	50	87	84	61	85	85
	3	37	19	49	62	63	79	77
	1	44	75	84	94	99	92	88
	2	44	75	90	89	74	93	92
	3	44	25	66	68	79	93	90
	1	50	75	86	93	92	98	89
	2	50	150	91	95	96	97	91
	3	50	19	74	67	58	95	88
	1	72	50	86	92	94	99	81
	2	72	200	94	96	93	100	89
	3	72	25	53	76	66	99	73
	1	113	75	94	99	100	100	92
	2	113	200	94	98	100	99	89
	3	113	75	93	99	100	96	84
	1	141	75	91	100	91	100	89
	2	141	150	76	100	95	100	85
	3	141	50	87	94	95	100	85
	1	190	150	92	97	100	100	94
	2	170	150	85	97	100	100	86
	3	170	150	74	88	84	98	88

a, b: For explanation see Table 3 footnotes a and b, respectively .

c: CTB-ELISA's E1CSFV, E1-Bac, E1-Bac-DIF, E1-Bac-dBC-DIF and E2-Bac are explained on Example 5. Test results are expressed as the percentage inhibition of a standard signal; <30% inhibition is negative, 30-50% inhibition is doubtful, >50% inhibition is positive.

TABLE 5. Comparison of CTB-ELISA's with CSFV strain 331 sera

strain	pig	DPI[a]	NPLA[b]	CTB-ELISA[c]				
				E1 CSFV	E1-Bac	E1-Bac DIF	E1-Bac dBC-DIF	E2-Bac
331	1	0	<12.5	0	0	0	8	0
	2	0	<12.5	4	5	0	0	0
	3	0	<19	4	13	0	0	0
	4	0	<12.5	0	14	0	0	0
	5	0	<12.5	5	11	11	0	0
	1	10	<12.5	0	13	0	14	13
	2	10	<12.5	0	11	0	0	13
	3	10	<12.5	0	20	16	24	31
	4	10	<12.5	0	29	0	9	26
	5	10	<12.5	0	24	7	38	18
	1	14	<12.5	0	34	9	34	8
	2	14	<12.5	2	18	0	0	36
	3	14	19	28	67	22	60	60
	4	14	19	35	77	37	60	10
	5	14	25	32	99	74	90	23
	1	17	19	7	84	53	69	0
	2	17	<12.5	23	0	0	0	4
	3	17	25	63	93	62	87	54
	4	17	37	55	82	36	80	0
	5	17	37	69	100	84	94	3
	1	21	37	57	84	100	50	39
	2	21	37	29	52	0	26	3
	3	21	100	76	93	100	96	96
	4	21	50	76	90	100	91	63
	5	21	75	65	ND	ND	ND	72
	1	28	75	73	95	100	96	96
	2	28	25	59	89	100	79	58
	3	28	300	78	100	100	100	100
	4	28	150	74	93	100	80	82
	5	28	100	72	98	100	100	91
	1	35	75	83	95	100	97	98
	2	35	150	80	98	100	100	96
	3	35	300	80	99	100	100	100
	4	35	200	81	ND	ND	ND	ND
	5	35	150	82	98	100	100	90
	1	42	150	81	98	100	96	99
	2	42	200	80	100	94	100	98
	3	42	300	79	94	100	100	100
	4	42	200	79	97	100	99	100
	5	42	150	80	99	100	100	90

a, b, c: See footnotes of Table 4.

TABLE 6. Comparison of CTB-ELISA's with CSFV strain Bergen sera

strain	pig	DPI[a]	NPLA[b]	CTB-ELISA[c]				
				E1 CSFV	E1-Bac	E1-Bac DIF	E1-Bac dBC-DIF	E2-Bac
Bergen	1	0	<12.5	0	2	0	0	0
	2	0	<12.5	0	0	0	0	0
	3	0	<12.5	0	2	0	0	0
	4	0	<12.5	0	7	0	0	0
	5	0	<12.5	0	4	0	2	0
	1	10	<12.5	0	0	6	0	27
	2	10	<12.5	0	5	0	0	4
	3	10	12.5	0	25	0	27	21
	4	10	<12.5	0	15	0	13	29
	5	10	12.5	0	14	0	6	21
	1	14	<12.5	0	51	32	18	12
	2	14	<12.5	8	12	9	10	4
	3	14	37	20	76	53	72	11
	4	14	12.5	0	55	8	77	18
	5	14	<12.5	0	17	9	10	0
	1	17	25	57	93	84	73	3
	2	17	<12.5	28	45	0	51	29
	3	17	75	75	100	78	90	0
	4	17	37	47	88	57	85	16
	5	17	12.5	23	54	11	55	0
	1	21	25	76	96	100	100	96
	2	21	19	62	78	54	72	67
	3	21	50	77	ND	ND	ND	63
	4	21	50	72	95	100	93	96
	5	21	50	51	80	97	88	0
	1	28	100	81	100	100	100	96
	2	28	37	80	97	100	100	81
	3	28	100	80	96	100	100	72
	4	28	50	81	100	100	100	100
	5	28	150	79	98	100	99	3
	1	35	150	84	98	100	100	100
	2	35	50	82	95	100	100	49
	3	35	100	79	100	100	100	66
	4	35	200	79	100	100	100	82
	5	35	200	78	98	100	100	21
	1	42	300	82	100	97	89	74
	2	42	300	81	98	100	100	49
	3	42	300	82	100	65	100	65
	4	42	200	81	98	92	100	74
	5	42	600	81	98	100	98	0

a, b, c: See footnotes of Table 4.

TABLE 7. Comparison of CTB-ELISA's with CSFV strain Henken sera

strain	pig	DPI[a]	NPLA[b]	CTB-ELISA[c]				
				E1 CSFV	E1-Bac	E1-Bac DIF	E1-Bac dBC-DIF	E2-Bac
Henken	1	0	<12.5	0	0	0	0	0
	2	0	<12.5	0	0	0	0	0
	3	0	<12.5	1	0	0	0	0
	4	0	<12.5	0	2	0	0	0
	5	0	<12.5	0	0	0	0	0
	1	10	<12.5	0	0	0	0	0
	2	10	<12.5	3	6	1	1	25
	3	10	<12.5	0	5	12	0	52
	4	10	<12.5	0	6	0	4	27
	5	10	<12.5	0	12	0	0	8
	1	14	<12.5	0	0	6	1	0
	2	14	<12.5	0	54	22	29	10
	3	14	50	5	57	67	100	20
	4	14	<12.5	0	7	0	34	0
	5	14	<12.5	0	12	10	9	0
	1	17	<12.5	0	0	0	7	0
	2	17	12.5	48	73	26	63	53
	3	17	75	75	100	94	100	35
	4	17	19	7	56	0	60	23
	5	17	12.5	0	29	0	16	15
	1	21	<12.5	29	0	0	0	0
	2	21	50	75	ND	ND	ND	ND
	3	21	300	84	ND	ND	ND	ND
	4	21	19	36	68	76	78	34
	5	21	50	63	83	61	82	32
	1	28	<12.5	0	0	0	0	0
	2	28	75	80	92	100	100	92
	3	28	600	80	99	100	100	82
	4	28	50	58	93	100	100	100
	5	28	50	79	99	100	100	100
	1	35	<12.5	22	13	13	0	1
	2	35	200	78	95	100	100	82
	3	35	300	78	100	100	100	75
	4	35	75	75	98	100	100	100
	5	35	150	80	98	100	100	97
	1	42	<12.5	17	12	9	0	0
	2	42	400	79	ND	ND	ND	ND
	3	42	400	79	ND	ND	ND	ND
	4	42	400	79	98	100	100	100
	5	42	300	82	98	100	100	90

a, b, c: See footnotes of Table 4.

TABLE 8. Comparison of CTB-ELISA's with CSFV strain Zoelen sera

strain	pig	DPI[a]	NPLA[b]	CTB-ELISA[c]				
				E1 CSFV	E1-Bac	E1-Bac DIF	E1-Bac dBC-DIF	E2-Bac
Zoelen	1	0	<12.5	0	0	0	0	0
	2	0	<12.5	0	5	0	0	0
	3	0	<12.5	14	4	0	0	0
	4	0	19	6	0	0	0	0
	5	0	<12.5	16	35	0	19	0
	1	10	<12.5	0	16	8	3	31
	2	10	<12.5	0	14	0	0	15
	3	10	<12.5	0	10	2	8	24
	4	10	19	12	8	0	0	22
	5	10	<12.5	0	27	27	12	24
	1	14	19	19	60	18	75	41
	2	14	<12.5	4	36	4	34	10
	3	14	<12.5	19	26	14	23	12
	4	14	25	26	91	40	92	39
	5	14	12.5	0	50	16	41	0
	1	17	37	61	96	76	91	64
	2	17	19	29	94	62	73	0
	3	17	12.5	23	41	16	45	4
	4	17	37	65	97	82	95	0
	5	17	37	48	90	60	75	0
	1	21	150	78	95	100	99	84
	2	21	19	68	89	100	94	58
	3	21	37	60	73	99	77	0
	4	21	75	75	92	100	95	46
	5	21	37	54	ND	ND	ND	57
	1	28	200	75	100	100	100	100
	2	28	150	76	100	100	100	89
	3	28	75	77	97	100	100	56
	4	28	300	79	97	100	100	84
	5	28	100	67	100	100	100	80
	1	35	400	82	100	100	100	100
	2	35	150	72	100	100	100	91
	3	35	200	81	98	100	100	60
	4	35	150	80	94	100	100	77
	5	35	100	79	99	100	100	89
	1	42	400	82	93	100	100	100
	2	42	200	67	99	100	100	78
	3	42	400	83	94	100	100	84
	4	42	300	82	99	100	100	86
	5	42	150	82	94	100	100	32

a, b, c: See footnotes of Table 4.

Claims

1. Nucleotide sequence corresponding to a classical swine fever virus (CSFV) genome or a part or a mutant thereof, characterised in that it comprises at least a part of the nucleotide sequence of the CSFV C-strain depicted in SEQ ID No. 1, or a complement or RNA equivalent of such nucleotide sequence.

2. Nucleotide sequence corresponding to a classical swine fever virus (CSFV) genome or a part or a mutant thereof, characterised in that it comprises a nucleotide sequence encoding at least the amino acid sequence 268–494 of the amino acid sequence depicted in SEQ ID No. 1, or a complement or RNA equivalent of such nucleotide sequence.

3. Nucleotide sequence according to claim 1 or 2, containing a mutation in the nucleotide sequence encoding amino acids 1–1063 of the sequence depicted in SEQ ID No. 1.

4. Nucleotide sequence according to claim 3, containing a mutation in the nucleotide sequence encoding amino acids 690–870.

5. Nucleotide sequence according to any one of claims 1–4, wherein said mutation is a substitution by a corresponding part of the genome of another pestivirus strain.

6. Nucleotide sequence according to any one of claims 1–4, wherein said mutation is a deletion, an insertion or a mutation resulting in a substitution of one or more amino acids encoded by the nucleotide sequence.

7. Nucleotide sequence according to any one of claims 1–4, wherein said mutation is an insertion of a heterologous nucleotide sequence altering the translation strategy of the CSFV nucleotide sequence or altering the processing of a polypeptide encoded by the CSFV nucleotide sequence.

8. Nucleotide sequence according to any one of claims 1–4, wherein said mutation is an insertion of a heterologous nucleotide sequence encoding a polypeptide inducing immunity against another pathogen.

9. Nucleotide sequenc according to any on of claims 1-4, wherein said mutation is an insertion of a heterologous nucleotide sequence encoding a marker polypeptide.

10. Polypeptide encoded by the nucleotide sequence according to any one of claims 1-9.

11. Pestivirus polypeptide corresponding to the amino acid sequence 690-1063 of SEQ ID No. 1 or part thereof, characterised in that it contains a mutation in one of the epitopes within amino acid sequences 691-750 or 785-870, said mutation altering said epitope.

12. Nucleotide sequence encoding the polypeptide according to claim 11.

13. Vaccine strain, the genome of which is derived from a full-length DNA copy, and/or an infectious transcript thereof, of the nucleotide sequence according to any one of claims 1-8.

14. Vaccine containing a polynucleotide having the sequence according to any one of claims 1-9 or 12, a polypeptide according to claim 10 or 11, or a vaccine strain according to claim 13.

15. Diagnostic composition containing at least a nucleotide sequence according to any one of claims 1-9, a polypeptide according to claim 10 or 11, and/or an antibody raised against a polypeptide according to claim 10 or 11.

16. Use of the nucleotide sequence TTTTCTTTTTTTT as a marker for CSFV C-strain derived sequences.

17. Method of diagnosis distinguishing pestivirus infected animals from vaccinated animals, said vaccinated animals being vaccinated with a pestivirus polypeptide or pestivirus strain containing a mutation in aminoacid sequence 268-494 or 690-1063 of SEQ ID No. 1, wherein a test sample is contacted with a pestivirus antigen corresponding to aminoacid sequence 268-494 or aminoacid sequence 690-1063 or part thereof and with an antibody directed against an epitope of said pestivirus antigen, which epitope is not functionally present in the mutated polypeptide or pestivirus strain used for vaccination.

18. Method according to claim 17, wherein said pestivirus antigen is a dimerised or multimerised polypeptid and part of said antibody is immobilised and another part f said antibody is labeled.

19. Method according to claim 17 or 18, wherein said pestivirus polypeptide and said antigen correspond to aminoacid sequence 690-1063, and said epitope is located between amino acids 785 and 870.

20. Method of determining the presence of a test substance capable of specifically binding with a binding site of a binding partner, in a sample, by means of competition of said test substance with a measurable amount of a reference substance capable of specifically binding with the same binding site of said binding partner, which comprises:
(1) contacting said sample with (a) the binding partner of said reference substance, said binding partner molecule containing at least two identical binding sites for said reference substance, (b) said reference substance bound to a solid carrier, and (c) said reference substance provided with a label;
(2) measuring the degree of binding of said label to said carrier.

21. Method according to claim 20, wherein said binding partner is a dimer or multimer of a binding partner to said reference substance.

22. Method of determining the presence of a test substance having at least two identical binding sites per molecule for specifally binding with a binding partner, in a sample, which comprises:
(1) contacting said sample with (a) said binding partner bound to a solid carrier, and (b) said binding partner substance provided with a label;
(2) measuring the degree of binding of said label to said carrier.

23. Diagnostic kit containing: (a) a reference antibody bound to a solid carrier, (b) said reference antibody provided with a label; and optionally (c) an antigen to said reference antibody containing at least two identical binding sites for said reference antibody; or a complex between said components (a), (b) and (c); as well as further components for carrying out a competitive immunological assay.

fig -1

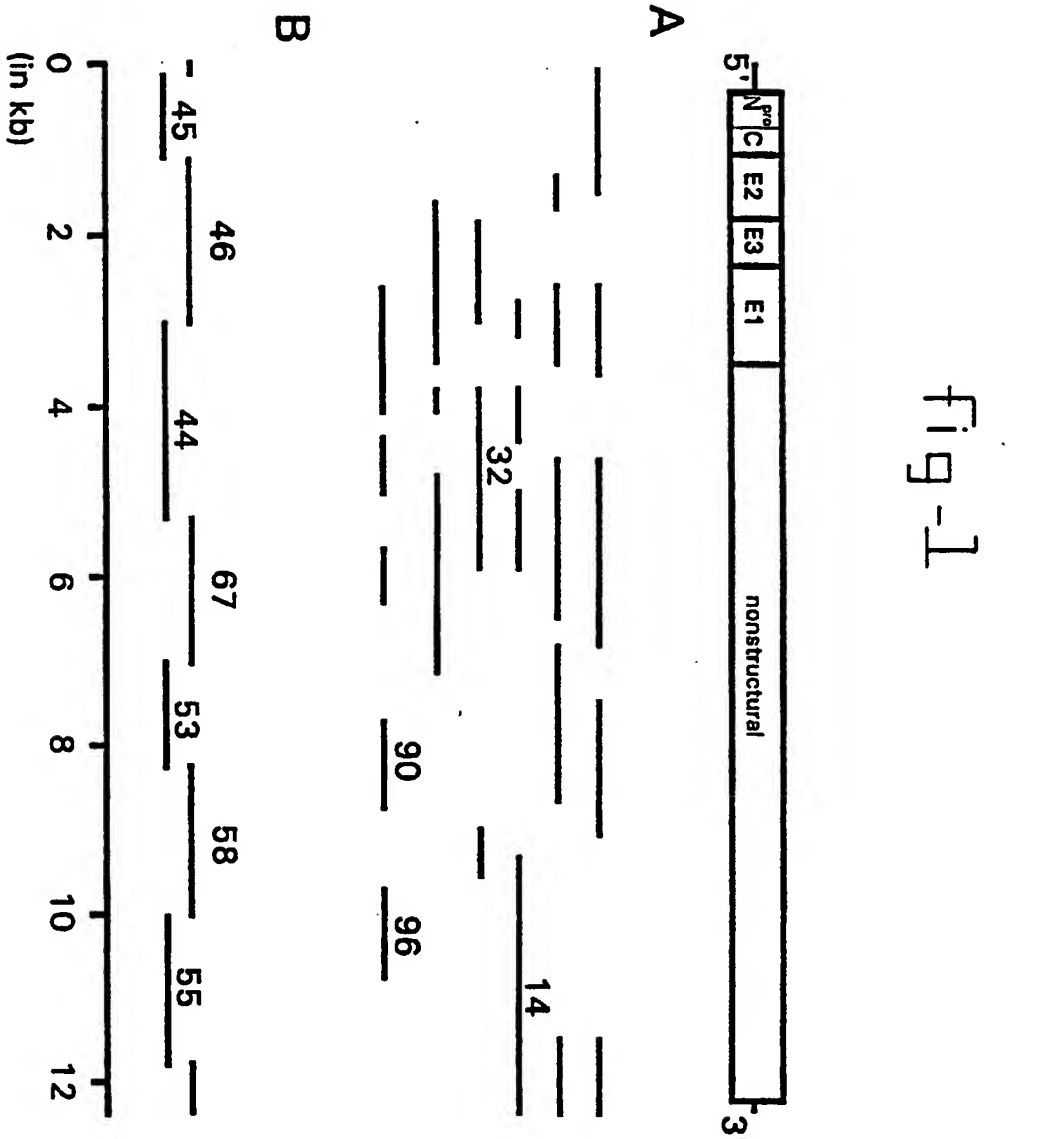
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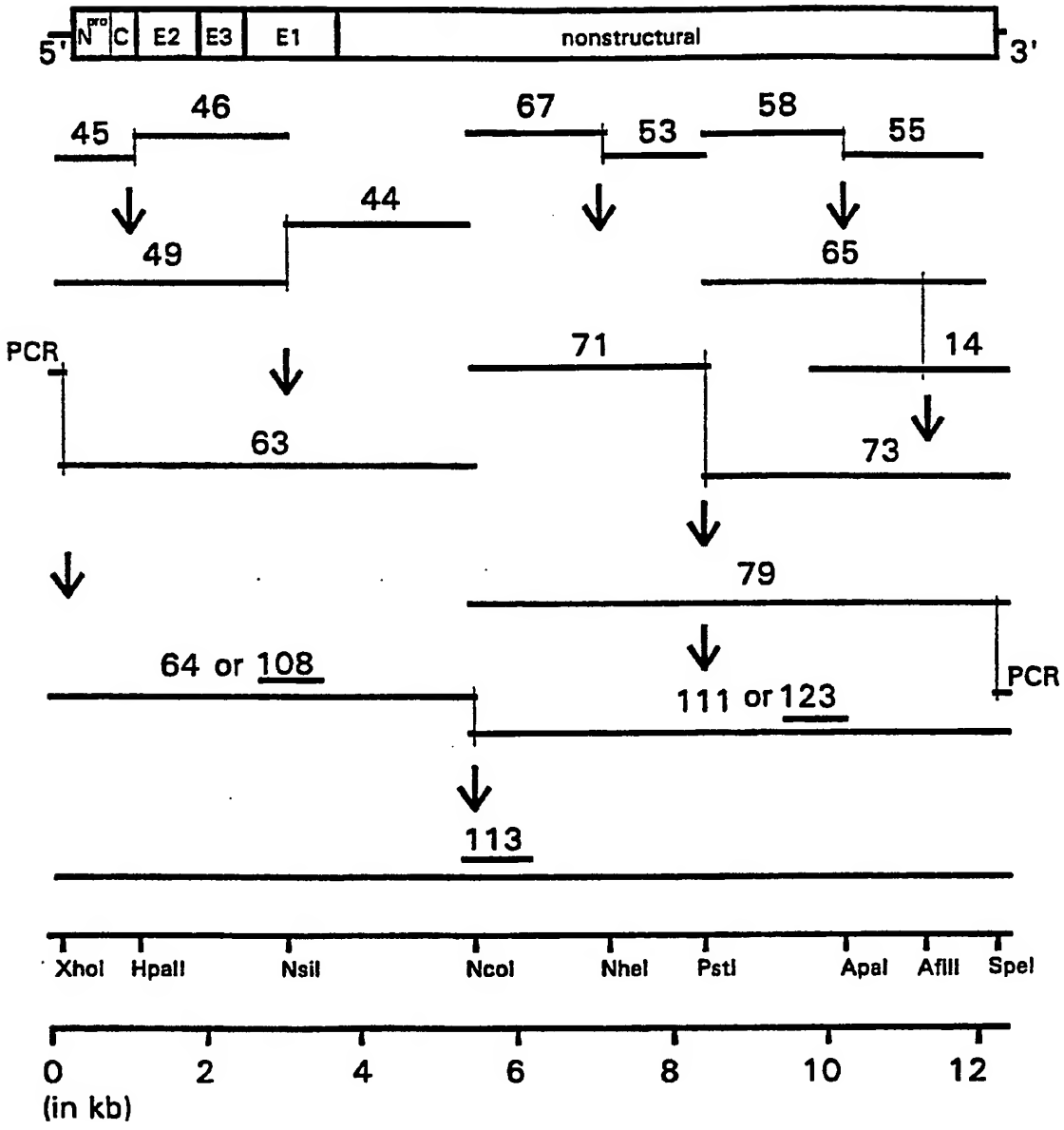
Fig-2A

```
Brescia     GTATACGAGG TTAGTTCATT CTCGTGTACA TGATTGGACA AATCAAAATC
Alfort      .......... ....C..T.. .....A...G AT......T. C.CT...-.T
C-strain    .......... .......... .....A.... C......... .........T

Brescia     TCAATTTGGT TCAGGGCCTC CCTCCAGCGA CGGCCGAGCT GGGCTAGCCA
Alfort      ..G....... CT.....AC. .......... .......AA. ..........
C-strain    .T........ .......... .......... .......... ..........

Brescia     TGCCCACAGT AGGACTAGCA AA-CGGAGGG ACTAGCCGTA GTGGCGAGCT
Alfort      ......T... .......... ..-....... .......... ..........
C-strain    ......T... .......... ..A....... .......A.. ..........

Brescia     CCCTGGGTGG TCTAAGTCCT GAGTACAGGA CAGTCGTCAG TAGTTCGACG
Alfort      .......... .......... .......... .......... ..........
C-strain    .......... .......... .......... .......... ..........

Brescia     TGAGCAGAAG CCCACCTCGA GATGCTATGT GGACGAGGGC ATGCCCAAGA
Alfort      ......CT.. .......... .......C.. .......... ..........
C-strain    .......... .......... .......C.. .......... .....-....

Brescia     CACACCTTAA CC-TAGCGGG GGTCGTTAGG GTGAAATCAC ACCATGTGAT
Alfort      .......... ..C.G..... .....C.... .......... .TT.......
C-strain    .......... ..C....... .....C.... .......... ....C.....

Brescia     GGGAGTACGA CCTGATAGGG TGCTGCAGAG GCCCACTATT AGGCTAGTAT
Alfort      ...G...... .......... .......... ........GC ..........
C-strain    .......... .......... C......... .......... ..........

Brescia     AAAAATCTCT GCTGTACATG GCACATG
Alfort      .......... .......... ....___
C-strain    .......... .......... ....___
```

Fig-2 B

```
Brescia     TGAGTGCGGG TGACCCGCGA TCTGGACCCG TCAGTAGGAC CCTATTGTAG
Alfort      ...CAT..T ..G...TT.. ..G..C..TA .......... .........A
C-strain    ...C..... .A.....G.. ....A..... C......... ..........

Brescia     ATAACACTAA ---------- ----TTTTTT ATTTATTTAG ATATTACTAT
Alfort      ......T... ---------- ---C..-A.. .A........ ...C..T...
C-strain    .......... TTTTCTTTTT TTTC...... .......... ......T...

Brescia     TTATTTATTT ATTTATTTAT TGAATGAGTA AGAACTGGTA CAAACTACCT
Alfort      .......... .......... ........C. ..T....... ..........
C-strain    .......... .......... .......... .......... T.........

Brescia     CATGTTACCA CACTACACTC A-TTTTAACA GCACTTTAGC TGGAAGGAAA
Alfort      .......... .......... .-.C...... .......... ....G.....
C-strain    ..A....... .......... .T........ .......... ..........

Brescia     ATTCCTGACG TCCACAGTTG GACTAAGGTA ATTTC-TAAC GGCCC
Alfort      ..-....... .......... .......... .....C.... ...--
C-strain    .......... .......... .......... .....-.... .....
```

Fig-3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PCR
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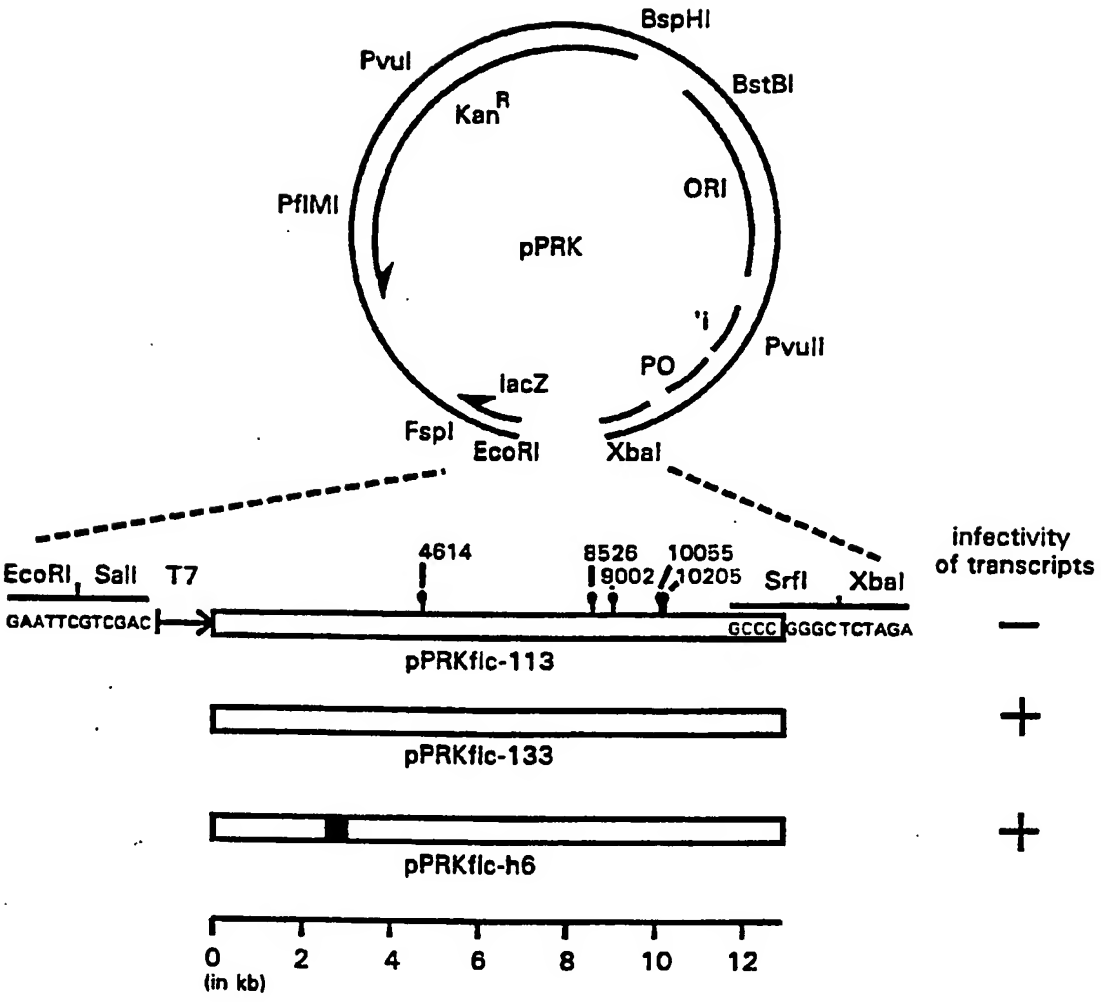
Fig-4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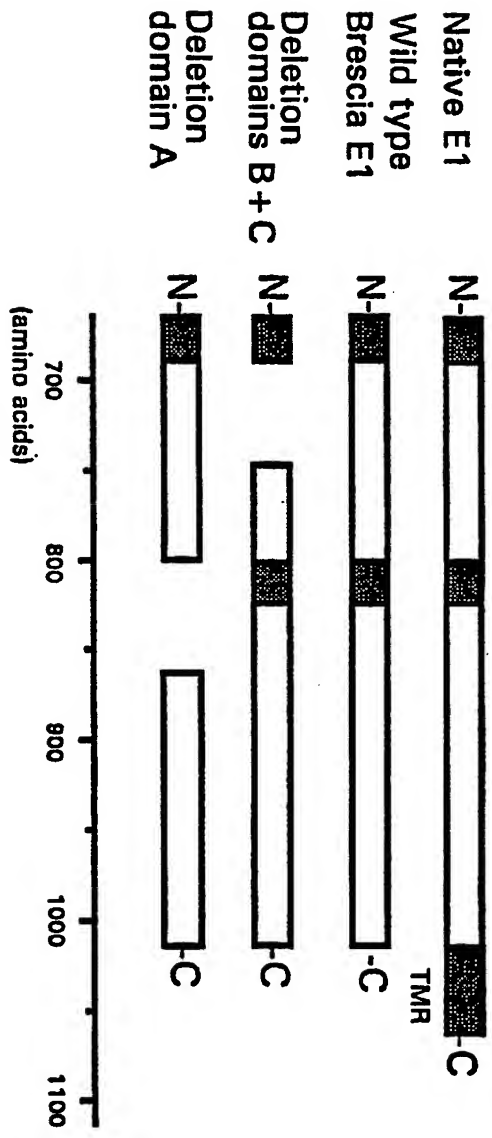
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Wild type Brescia E1
Deletion domains B+C
Deletion domain A
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-C
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800
900
1000
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(amino acids)

fig - 5

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/NL 95/00214

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/40 C12N7/02 G01N33/569 A61K39/187 C12Q1/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP,A,0 322 990 (DE STAAT DER NEDERLANDEN VERTEGENWOORDIGD DOOR DE MINISTER VAN ...) 5 July 1989 see page 7, line 31 - page 8, line 5; claims 1,7,11	20-23 18
Y	EP,A,0 351 901 (CENTRAAL DIERGENEESKUNDIG INSTITUUT) 24 January 1990 cited in the application see the whole document	1-4, 6-15,17, 18
Y	WO,A,91 00352 (CENTRAAL DIERGENEESKUNDIG INSTITUUT) 10 January 1991 cited in the application see the whole document	1-4,6-15

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
2 November 1995	16.11.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Gurdjian, D

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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